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THE UNIVERSITY OF ALBERTA

SOMITIC CONTRIBUTION TO VERTEBRAL FORMATION

BY

SUSAN JENNIFER HIGGINS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ANATOMY AND CELL BIOLOGY

EDMONTON, ALBERTA

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To Friends

ABSTRACT

The somitic involvement in formation of the vertebral column was examined in the chick-quail chimera model. Single cervical somites from quail donor embryos were transplanted into similarly staged chick host embryos. Following further incubation, serial sections of variously staged embryos were stained for the Feulgen reaction to distinguish the two cell populations.

Quail cells were generally located within a delimited region in portions of two adjacent vertebrae, as well as in paravertebral muscle, dermis, spinal cord meninges, and general connective tissue. It was concluded that a single vertebra is a composite structure, being derived from cells of two consecutive pairs of somites. There also appeared to be a range of dispersion among the various subpopulations of the somite. Myogenic quail cells were present within several vertebral levels; chondrogenic cells remained primarily within the originally defined boundaries of the somite and sclerotome.

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LIST OF ABBREVIATIONS FOR FIGURES

A	Aorta
C	Cervical
U Ce	Centrum
Ch	Chick
CP	Costal Process
CT	Connective Tissue
D	Dermatome
Di	Disc
Dm	Dermatomyotome
E	Ectoderm
En	Endoderm
F	Intervertebral Foramen
G	Spinal Ganglion
IC	Intersomitic Cleft
IF	Intrasclerotomic Fissure
IM	Intermediate Mesoderm
IN	Inferior (Caudal) Notch of Pedicle*
IP	Inferior (Caudal) Articular Process*
IV	Intersomitic Vessel
J	Articular Joint
L	Lamina
LP	Lateral Plate Mesoderm
M	Myotome
Mu	Muscle

LIST OF ABBREVIATIONS FOR FIGURES (continued)

My	Myocoel
N	Notochord
NT	Neural Tube
P	Pedicle
PS	Perinotochordal Space
PT	Perichordal Tube
Q	Quail
S	Somite
SC	Spinal Cord
SN	Spinal Nerve
SP	Superior (Rostral) Articular Process*
SpC	Spinal Canal
SpP	Spinous Process
St	Sclerotome
T	Transverse Foramen
TCP	Tip of Costal Process
TP	Transverse Process

* For identification of certain landmarks, the terms "rostral" and "caudal" have been replaced with "superior" and "inferior", respectively, as the latter two terms are more commonly employed in the discipline of Anatomy.

I. INTRODUCTION

To explain abnormal development in any system, it is necessary first to have an understanding of normal developmental events. Underlying principles can then be applied in tracing how deviations from the normal may result in the various anomalies which are observed. Certain abnormalities of the vertebral column, such as scoliosis and costovertebral dysplasia, have been linked to possible disturbances in the early morphogenesis of this system (Tsou *et al.*, 1979; Gassner, 1982). Accurate identification of the precursive structures would seem to be a basic element in the process of determining the developmental progression leading to aberrations of the vertebral pattern.

Formation of the vertebral column has been an extensively studied aspect of morphogenesis for more than a century (Verbout, 1976). The numerous theories which have been put forth can be generally separated into two opposing positions. Many investigators have formulated different variations of the "Resegmentation Theory", initially proposed by Remak (1855: cited in Verbout, 1976). It was claimed that the original segmental arrangement of vertebral precursors (sclerotomes) was lost as adjacent tissues merged with each other. Rostral and caudal borders of the definitive vertebrae later formed at new positions resulting in a shift of one-half segment (Piiper, 1928; Williams, 1942; Sensenig, 1949). A smaller number of researchers, including some who have reported more recent studies, concluded that resegmentation of the original units did not occur (Baur, 1969: cited

in Verbout, 1976; Dalglish, 1985; Verbout, 1985). The origin of the cells which form a single vertebra remains an area of contention.

The evidence put forth in support of resegmentation was dependent largely upon maintenance of relative positions of adjacent structures. In particular, proponents of resegmentation have assumed that the sclerotome cells retained the same positional relationships with the intersomitic arteries, spinal nerves, and myotomes throughout development. Investigations of other systems have demonstrated that embryonic tissues do not necessarily remain in the same locations relative to surrounding structures. Arguments used to support resegmentation could be challenged on the basis of reliability of cell identification due to the methodology employed: examination of serial sections of sequentially staged normal embryos.

From examination of the literature, it was apparent that the following questions had not been answered:

1. What is the cellular contribution of a single somite to the vertebral column?
2. What are the relationships of sclerotome cells from a single somite to adjacent structures during the early phases of vertebral formation?

In tracing the cell lineage of a body structure, the primary concern must be the certainty with which precursor or derivative cells can be identified. The long-standing theory of somatic origin of limb musculature has been negated recently in favor of somitic origin (Chevallier *et al.*, 1977; Christ *et al.*, 1977) through utilization of the chick-quail chimera model developed by Le Douarin (1973). Without

the marker system somitic premyoblasts were indistinguishable from the local limb bud mesenchyme. None of the studies of vertebral development have utilized permanent cell markers. The apparent complexity of vertebral formation would seem to indicate the need for an experimental study which uses a cell marker system.

This study was undertaken to re-examine the question of somitic involvement in vertebral formation, and to investigate the distribution patterns of sclerotome cells in the young embryo. To this purpose, use of a permanent cell-marker system was employed to permit later identification of descendent cells from heterospecific single somite transplants.

For study of these events, the primary experiment involved removal of single somites from approximately two-day old chick embryos, and replacement with single somites from similarly staged quail embryos. Avian embryos (chick host and quail donor) were selected as the models for this study for several reasons:

1. The similarity of avian embryonic development to that of mammals (LeDouarin, 1985);
2. The similarity in size and developmental rates of early chick and quail embryos, thus facilitating tissue transplants between the two animals;
3. The availability of a specific staining technique which permitted distinction histologically of quail cells from chick cells in all tissues and throughout all developmental stages (LeDouarin, 1973).

The mid-cervical column was chosen as the focal region for examination since it consists of many nearly identical vertebrae in birds. These vertebrae also have many features in common with mammalian cervical vertebrae. Additionally, the cervical vertebrae are the first to develop in birds, and they are easily accessible for removal and study.

Following further incubation, embryos were fixed at various stages up to stage 36 of Hamburger and Hamilton (1951) (H & H). Serial sections of the operated region were stained by the Feulgen technique for identification of the quail cells (LeDouarin, 1973). The cellular derivatives from a single somite were then localized within the axial structures. Somitic contribution to the vertebrae was clarified by the generation of serial reconstructions which illustrated the regions populated by quail cells. It is believed that the results of this study have provided evidence that a single vertebra is formed of cells derived from two consecutive pairs of somites.

II. REVIEW OF RELATED LITERATURE

1. Vertebral Column of *Gallus domesticus*

A. Vertebral Column as a Whole

The vertebral column of the mature domestic fowl consists of 40 to 42 bones regionalized as follows: 14 cervical, 7 thoracic, 14 lumbosacral, and 5 to 7 coccygeal (Kaupp, 1918; Nickel *et al.*, 1977). Within each of these regions specializations are apparent in the individual vertebral elements as well as in the articulations between adjacent vertebrae.

B. Cervical Vertebrae

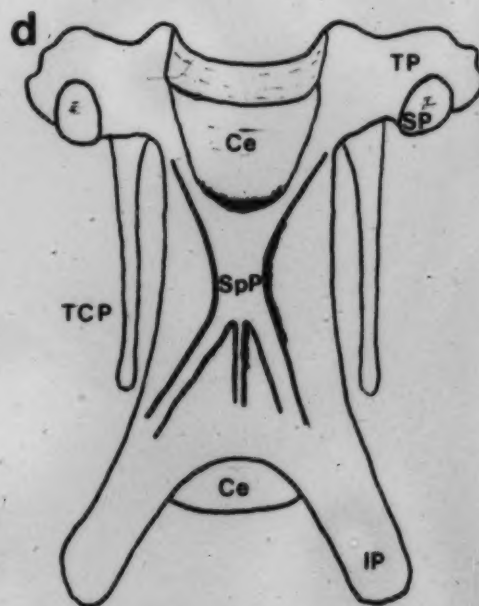
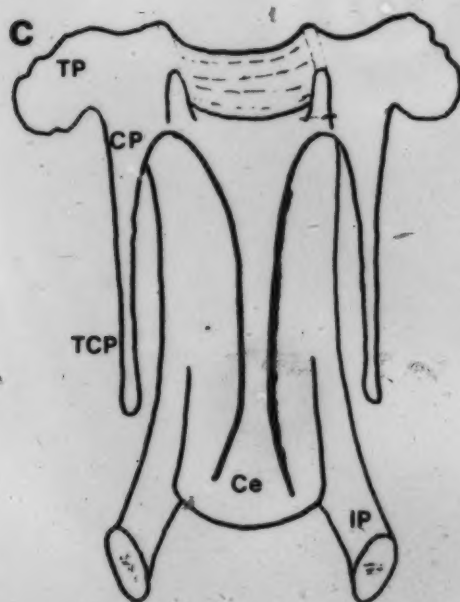
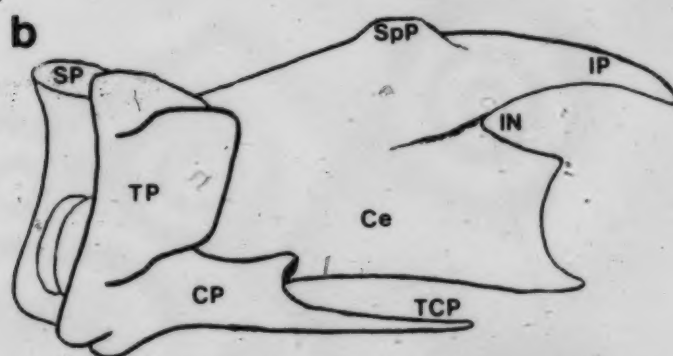
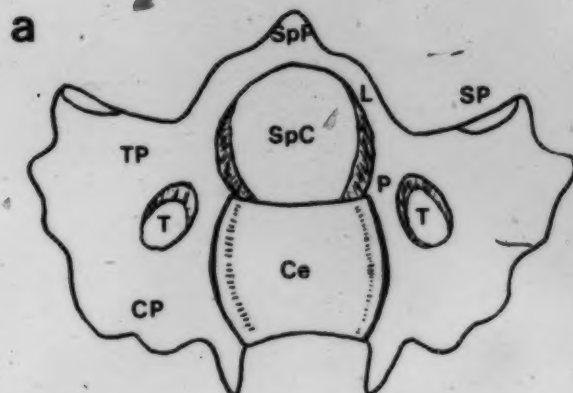
The first two cervical vertebrae are uniquely structured in order to optimize movement between the skull and vertebral column (Kent, 1978). The first cervical vertebra is a narrow bony ring which articulates with the single occipital condyle, and with the odontoid process and the superior articular processes of the second cervical vertebra, the axis (Kaupp, 1918; Nickel *et al.*, 1977). The axis consists of a short vertebral body and a neural arch. It possesses the rostrally projecting odontoid process, as well as superior and inferior articular processes, and dorsal and ventral (hemal) spinous processes (Kaupp, 1918; Nickel *et al.*, 1977; Wake, 1979). With the exception of the atlas, each cervical vertebra is comprised of a vertebral body (centrum) and vertebral or neural arch which encloses the spinal canal.

In the remaining cervical vertebrae, the body of each successive vertebra is longer than that of the preceding one (Kaupp, 1918).

The articular surfaces of the centra are heterocelous (i.e. saddle-shaped, or concave transversely and convex dorsoventrally on the rostral surface, with the caudal surface being the opposite) (Nickel *et al.*, 1977; Kent, 1978). Between adjacent centra are articular intervertebral discs (Koch, 1973). A ligament through the discs connects adjacent centra, but no notochord remnant (nucleus pulposus) is present (Nickel *et al.*, 1977). Projecting ventrally from the centrum are paired ventral or hemal spines which may be fused into a single ridge (Nickel *et al.*, 1977). The ribs of the cervical region are rudimentary and are fused to the vertebrae forming the costal processes (Romanoff, 1960). Projecting from the neural arch are a number of processes. The superior and inferior articular processes form diarthrodial gliding joints with corresponding facets of adjacent vertebrae. Each transverse process is fused with the costal process and encloses a transverse foramen allowing passage of the vertebral vessels. This foramen is present only in the cervical vertebrae of birds and mammals (Kent, 1978). The dorsally projecting spinous processes are less conspicuous in the middle cervical vertebrae (McLeod *et al.*, 1964). The superior and inferior notches of adjacent vertebrae combine to form the intervertebral foramina which transmit the spinal nerves (Kaupp, 1918). The neural arches do not completely protect the spinal canal dorsolaterally, the exposed regions being covered by intervertebral ligaments. Illustrations of a typical mature cervical vertebra have been presented in Figure 1.

Figure 1. Illustrations of a cervical vertebra of a mature chick

Rostral (a), lateral (b), ventral (c), and dorsal (d) views of an ossified middle cervical vertebra are presented. Note the elongated centrum and the inferiorly projecting tip of the costal process noticeable in the lateral view. The transverse foramen can be seen in the rostral view.



2. Formation of Vertebrae

The general sequence of events in vertebral formation would appear to be common among vertebrates (Williams, 1959), and according to Parke (1982) includes three phases. The mesenchymal or membranous phase involves formation of the perichordal tube, a sheath of mesenchymal cells which surround the notochord. Chondrification then begins and during the cartilaginous phase the individual vertebrae and the intervening intervertebral discs are discernable. Finally ossification takes place and the vertebral column assumes its mature form in the skeletal phase. These events occur sequentially throughout the column, but in chicks each successive process is initiated in the rostral (cervical) regions and progresses caudally (Romanoff, 1960).

It has been generally accepted that the vertebral rudiments are formed from chondrification of sclerotome cells. The manner in which the groups of cells merge to produce the definitive form, however, has been an area of controversy. The formation of the vertebral column would seem to be somewhat complex, involving various cellular interactions which apparently must be synchronized with the development of other systems. For the purpose of reviewing the pertinent literature, this process has been divided into sections based upon the chronological sequence of events.

A. Somites

The cell population presumed to be involved in vertebral formation is the sclerotome, one of the derivatives of the somites. Following gastrulation, in the most medial portion of the mesoderm (the segmental

plate) a series of transverse furrows appear commencing near the rostral end of the embryo and progressing caudally. The furrows are the intersomitic clefts and they delineate the rostral and caudal boundaries of discrete units of cells: the somites. In the chick embryo, approximately 50 pairs of somites eventually form (Romanoff, 1960; Bellairs, 1985).

Beginning at approximately 22 hours of incubation, the first intersomitic cleft forms as the rostral boundary of the first persisting somite, more rostral somites forming later as rudimentary and transitory structures (Williams, 1910; Menkes and Sandor, 1977). Caudal somites are separated from the segmental plates at an average rate of one pair every 75 minutes (Menkes and Sandor, 1977). After the first three sets of divisions have taken place, lateral boundaries form separating the somites and segmental plate from the intermediate and lateral plate mesoderm (Williams, 1910). Cell processes are present between the first few somites until about the 6 or 7 somite stage at which time complete separation of the somites occurs (Romanoff, 1960). Some of the factors which have been implicated as possibly being involved in somite segmentation include an increase in cell-cell adhesivity (Bellairs *et al.*, 1978; Cheney and Lash, 1984), contractile filaments (Chernoff and Lash, 1981), and extracellular matrix materials such as collagen (Hay, 1973; Bellairs, 1979) and fibronectin (Lash and Ostrovsky, 1984).

It has been suggested that the *in vivo* morphology of intact somites is continuously altered by the changing shapes of surrounding structures: adjacent somites, neural tube, and notochord (Packard and

Jacobson, 1979). Immediately following removal of a somite, the two adjacent somites bulge into the vacated space, suggesting that compressive forces contribute to the rectangular appearance of the somites viewed from the dorsal surface (Packard and Jacobson, 1979).

After each somite has formed, continued mitotic activity produces a corresponding increase in the size of the somite. Although the rostral somites are always larger and more advanced than successive somites, there is a progressive increase caudally in the size of each newly-formed somite up to the thirtieth somite (Williams, 1910). The later-forming somites are also apparently more mature at formation than the earlier ones, and omit or speed up some of the initial developmental processes (Williams, 1910; Romanoff, 1960). There is thus less of a maturity gap between somites of different levels.

Several theories have been put forth to explain the triggering mechanism of somitogenesis. Although many of the relevant processes have been investigated, controversy still exists regarding the factors which stimulate somite formation. Bellairs (1985) has suggested the existence of pre-somite clusters in the presumptive somite area, these units possibly programmed for somite formation which may be stimulated by ingressing cells from the primitive streak (Ooi et al., 1986). Other mechanisms for control of somitogenesis have been hypothesized, as reviewed by Bellairs and Portch (1977), and Bellairs (1980, 1985).

B. Somitic Derivatives

Eventually the cells of the ventromedial somite wall separate from the rest of the somite to form a mesenchymal mass of tissue, the

sclerotome, while the remaining dorsolateral portion, the dermatome, retains its epithelial structure (Langman and Nelson, 1968). Results of Gallera's (1966) study indicated that association of the ectoderm with the dorsal region of the somite produces differentiation of the dermatome: sclerotome formation apparently is a result of interaction between the ventral portion of the somite with the endoderm. If prior determination of these two regions is present, it is reversible. Somitic derivatives which were either entirely epithelial or entirely mesenchymal in nature were produced following culturing of somites surrounded by either only ectoderm or only endoderm, respectively.

The myotome, precursor of the voluntary musculature, forms from the overlying dermatome commencing in the dorsomedial angle (Langman and Nelson, 1968; Miki and Mizoguti, 1982). The more superficial cells of this layer, now the definitive dermatome, gradually "lose their epithelial continuity and move under the surface ectoderm to form the dermis" (Langman and Nelson, 1968, 220).

The three primary derivatives of the somite give rise to at least three types of tissues: sclerotome forming cartilage, myotome producing muscle, and dermatome forming dermal connective tissue. The differentiation of these tissues is affected by inductive influences of adjacent structures. In particular, the notochord and neural tube have been shown to influence cartilage formation (reviewed in Hall, 1977, 1986) (discussed below). Culturing of ectoderm with somites has been shown to promote formation of general connective tissue, possibly indicative of the inductive interaction between the dermatome and the overlying ectoderm in ovo (Swalla and Solursh, 1984).

Information regarding the early state of determination of somitic cells appeared to be somewhat contradictory. *In vitro* experimentation has indicated that somitic cells possess a chondrogenic bias (Hall, 1977, 1986). Grafts of somites into developing wing buds, however, produced muscle tissue more frequently than cartilage (Wachtler et al., 1982). The latter group of investigators concluded that myogenic determination of somitic cells preceded chondrogenic determination, but acknowledged that the cells could be redetermined to differentiate along another cell line.

From the literature it would appear that the cells of the newly formed somite are pluripotent with respect to their ability to form cartilage, muscle, or connective tissue. As further evidence of this capacity, Holtzer and Detwiler (1953) in *urodeles*, and Strudel (1953: cited in Hall, 1977) in chicks, observed the formation of a secondary set of neural arches in association with the grafting of an accessory neural tube into the dorsolateral face of the somite (presumptive dermatome). The potency of the cells would seem to be of critical significance in regard to somite orientation in transplant experiments.

C. Sclerotome Dispersal

As the somite begins to differentiate, distinctive changes occur in the two cell regions. In the dermatome, apical junctional complexes become more extensive and the cells undergo further elongation. The developing sclerotome region, however, shows a reduction in the number and extent of apical junctions and there is an expansion of the intercellular spaces (Solursh et al., 1979). The sclerotome cells also

demonstrate numerous filopodia which are often sites of cell-cell focal junctions (Trelstad *et al.*, 1967). Although changes in the cellular morphology have been well documented, the events producing these changes have not been determined.

i. Mechanisms of Dispersal

Upon examination of the possible mechanisms of sclerotome dispersal, basically two opposing theories have been put forth. One group of investigators believe that the process involves directed active migration of the cells medially from the somite to surround the notochord and later the neural tube (Ebendal, 1977; Trelstad, 1977; Chernoff and Lash, 1981; Chernoff, 1985). The second opinion is that the final positions of the cells are achieved passively or through random movement of the cells, effectively diffusing from an area of high concentration to areas of lower concentration (Flint, 1977; Gasser, 1979; Solursh *et al.*, 1979). Each of these positions has some supportive evidence.

Solursh *et al.* (1979) suggested that hydration of hyaluronic acid resulted in expanding the intercellular spaces. Movement of sclerotome cells into this space, they believed, was influenced by compressive forces of adjacent tissues and possibly by traction forces of cells at the medial border which exhibited lamellipodia in association with the notochord. This theory concurred with that of Gasser (1979) who proposed that the apparent cell movement observed was due to relative positional shifts of structures resulting from asynchronous growth. With expansion of the embryo, the dermamyotome was shown to retain its

subectodermal position, apparently leaving a trail of sclerotome cells behind it. Neither Gasser (1979) nor Solursh *et al.* (1979) placed any significance on individual cell migration during sclerotome dispersal.

Studying the same phenomenon in chick embryos, Ebendal (1977) noted that cells possessed numerous pseudopodia, often in association with matrix fibrils which appeared to be radially oriented about the notochord. The morphology of sclerotome cells is consistent with that of other cells undergoing active locomotion (Chernoff, 1985). Cilia have also been observed on the "trailing" edges and filopodia on the "leading" edges of these cells (Trelstad *et al.*, 1967). It has further been shown that treatment of cells with a microfilament-disrupting chemical results in the arrest of sclerotome dispersal, again suggesting active movement of the cells (Chernoff and Lash, 1981).

Cultured sclerotome cells are highly motile on various types of substrata (Bellairs *et al.*, 1980; Sanders and Prasad, 1983), and it has been noted that the presence of some focal contacts in different types of cultured mesoderm cells does not seem to inhibit cell motility (Sanders, 1984, 1986a). The behavior of cultured cells, however, may not necessarily be indicative of cell behavior *in vivo*, as a possible alteration of plasma membrane constituents following explanting of cells to culture conditions has been suggested (Stern and Keynes, 1987).

Observation of cell behavior following transplants of quail somites into chick limb buds has indicated a possible distinction in motility of the myogenic and chondrogenic cell populations (Wachtler *et al.*, 1982). Quail myotubes were found mixed with chick cells at the

implant location and distally in the limb. Chondroblasts, however, were observed only at the site of the graft. The authors had assumed normal migratory behavior of chondrogenic cells when adjacent to the axial organs, and so concluded that the non-migratory observations in their study were attributable to environmental differences. It should be noted, however, that the heterotypic environment did not affect the migratory behavior of the myogenic cells, nor did it affect differentiation of the cells into myoblasts or chondroblasts.

A number of factors may be involved in the dispersal of sclerotome cells. The embryo does grow in size and it is possible that hydration of hyaluronic acid may further enlarge the intercellular spaces. Sanders (1986a) has suggested that the cells may be moving in clusters, these being maintained by the focal contacts; Solursh *et al.* (1979) proposed that a sheet-like movement of cells may occur, with force generated by the cells at the notochordal boundary. The direction of movement may be controlled by contact guidance of the matrix fibrils (Ebendal, 1977), contact inhibition (Ebendal, 1977), galvanotaxis (Erickson and Nuccitelli, 1984), chemotaxis, or possibly by an adhesive gradient. It is also possible that several, or none of these factors are operating and/or that some other mechanism is in effect.

ii. Pattern of Dispersal

Even though the somites of different levels vary somewhat in size and shape throughout development, the general pattern of sclerotome dispersal from each somite is quite similar. Williams' (1910)

description of the development of the tenth chick somite has been summarized as an example of the progression of the somitic mesoderm.

From an initial two-dimensional pentagonal shape, an extension of sclerotome cells projects toward the notochord from the ventromedial border of the somite producing a more triangular configuration by 17 somites (H & H stage 12). A portion of the notochordal region extends further ventrad for approximately one-third to one-fourth of one somite length. This observation was noted for each of somites 2 to 15 at which level the intersomitic clefts that follow become vertical. The rostral segments thus appear to be more oblique than transverse in orientation.

At the 25 somite stage (H & H stage 15) two regions in the sclerotome can be discerned: a medial less dense region, and a lateral more dense area. The notochordal portion lies in a coronal plane as the dorsal aortae have fused into the median aorta which is directly ventral to the notochord. By 30 somites (H & H stage 17) the dermatome has separated from the sclerotome which is coming into contact with cells of adjacent sclerotomes and now contains some blood vessels.

At 40 somites (H & H stage 19 to 20) the spinal ganglion has formed and is surrounded by mesenchyme. The sclerotome of this segment can be delineated only by the locations of the intersomitic vessels.

"No portion of this [perichordal] sheath can be assigned with accuracy to any particular somite or sclerotome, for we have seen that the rostral ends of the second to the fifteen segments extend far forward under the preceding sclerotomes." (Williams, 1910, 83)

This observation has implications regarding the foundations of vertebral formation theories, discussed later. The band of cells

between the aorta and the notochord has thickened, and some cells are present between the notochord and neural tube. The perichordal tube of sclerotome cells is continuous and homogeneous. More laterally, the sclerotome is dense rostrally and caudally with a region of less dense tissue between: the intrasclerotomal fissure.

At 48 to 52 somites (approximately H & H stage 23), the axial mesenchyme is denser and the lateral rostral and caudal condensations are continuous with a midsegmental condensation in the perichordal tube. With further development, the cartilaginous rudiments of the various vertebral elements begin to form in the specific regions of this sclerotomal mass (Williams, 1910).

D. Induction of Vertebral Anlagen

Development of the sclerotome into the definitive vertebral column occurs apparently as a result of two general processes. The sclerotome cells may be predetermined to form cartilage although the means by which they acquire this bias are unknown (Hall, 1977, 1986). The axial organs (spinal cord and notochord) impart an inductive influence on the cells resulting in chondrogenesis. Morphogenesis of the individual vertebrae may be the result of a number of interactions between the sclerotome and the adjacent tissues.

1. Chondrogenesis

Culturing of somites has provided information as to the role of the neural tube and notochord in cartilage formation. Explants of somites with either of the axial organs consistently produce cartilage,

whereas culturing of somites alone produces cartilage only in the appropriate medium, or after a particular stage of development (Hall, 1977, 1986). Action of an inducer present in the extracellular matrix formed by the notochord and ventral neural tube is thus indicated (Hall, 1986). Although the nature of the inducing agent(s) has not been elucidated, Vasan (1983) has shown that large proteoglycan aggregates in the perinotochordal material may be involved in initiating chondrogenesis, type II collagen having only a permissive role (Hall, 1986). Cartilage induction may be mediated through a reduction in intracellular cAMP levels in the somites (Vasan, 1986).

11. Morphogenesis

The necessary presence of the notochord and neural tube/spinal cord for vertebral formation in chicks has been demonstrated by Strudel (1967). Removal of the notochord resulted in formation of an unsegmented cartilaginous gutter ventral to the spinal cord. Excision of the neural tube, on the other hand, produced a continuous cartilaginous rod around the notochord and complete absence of neural arches. If both organs were removed, no vertebral elements formed. Operation of two possibly interactive but independent mechanisms may be taking place in forming the vertebral centra and the neural arches.

a. Centra. The notochord would seem to function as the focal axis for formation of the centra and intervertebral discs. Explants of notochord with somites have resulted in the development of cartilaginous rods encircling the notochord (Williams, 1942; Strudel,

1967). Deformation of the notochord by chemical means produced various abnormalities in the centra (Strudel and Gateau, 1971: cited in Hall, 1977). The cellular interactions that result in alternation of centra and intervertebral discs, however, is less well understood. Strudel (1967) has suggested that normal segmentation of the centra may be related to regression of the notochord, which in turn may be dependent upon the presence of ventral nerve roots (cited in Hall, 1977). This relationship has yet to be demonstrated experimentally.

b. Neural Arch. The presence of a normal neural tube is apparently necessary for the formation of neural arches. Separation of consecutive arches would seem to be dependent upon the presence of the spinal ganglia, as the excision of neural crest cells in chick embryos resulted in fusion of the neural arches (Strudel, 1967). In studying amphibian embryos, Detwiler (1934) replaced a few rostral somites with a greater number of smaller caudal somites. The number of supernumerary neural arches that formed corresponded exactly with the number of extra ganglia. Flint (1977) noted a correspondence between fused dorsal root ganglia and fusion of the developing vertebrae in mutant mouse embryos. Flint (1977) theorized that the nervous tissue mechanically pushed the sclerotome cells out of the way, generating a loose-cell zone and preventing chondrogenesis in that region. Holtzer (1952) demonstrated in amphibians that the size of the neural arch was positively correlated with the size of the neural tube. Holtzer (1952) suggested that the neuroblasts may prevent chondrogenesis by the release of an inhibitory substance. This last possibility would seem

to be in conflict with evidence that the neural tube promotes chondrogenesis, although it may be that timing of these interactions is involved, or that regional specificity of the neural tube exists.

F. Regional Diversity Within the Sclerotome

Numerous descriptive studies have noted that spinal ganglia and spinal nerves always form in the rostral portion of the sclerotome (Williams, 1910; Piiper, 1928; Williams, 1942; Sensenig, 1949; Flint, 1977; Dalglish, 1985; Verbout, 1985). Experimental evidence put forth by Keynes and Stern (1984) has shown that motor axons from the spinal cord grow preferentially through the rostral sclerotome half. Anterocaudal rotation of the neural tube by 180° did not alter the outgrowth of nerve axons; 180° rotation of the segmental plate resulted in the axons growing through the original rostral half of the sclerotome. It was also noted that mediolateral reversal of the segmental plate did not influence neurite growth patterns.

It has been demonstrated that neural crest cells destined to form the dorsal root ganglia migrate initially between the dermamyotome and sclerotome, and then into the sclerotome, but only into the rostral half (Rickmann *et al.*, 1985). As removal of the neural crest did not influence the direction of motor axon growth, it was concluded that the growth pattern of the neurites is not influenced by the presence of spinal ganglia, but must be related to some inherent differences in the sclerotome cell subpopulations. A possible inhibitory effect of the caudal sclerotome region on axon outgrowth has been suggested by the findings of Lewis *et al.* (1981) that motor neurites were evenly

distributed along the length of the neural tube when somitic tissue was destroyed by irradiation.

A distinct difference between rostral and caudal sclerotome cells in their ability to bind peanut agglutinin has been shown by Stern *et al.* (1986). The caudal sclerotome half was intensely labelled with the lectin, whereas the rostral half was only lightly labelled. The distinction was not observed prior to sclerotome formation in the somites.

Based upon the results of the previously mentioned studies it would appear that the differences between the two regions of the sclerotome are present prior to segmentation. Recent information has suggested that at least some of the differences between these two populations of cells may develop approximately 12 hours prior to formation of the somite (Bellairs *et al.*: cited in Stern *et al.*, 1986). Additionally, the specific properties would seem to be irreversible, and the cells are apparently unable to reorganize to form the original pattern. There exists some contradictory evidence which would question the latter conclusion. Menkes and Sandor (1977), and Stern and Keynes (1986) have shown that somitogenesis precedes normally after mechanical mixing of cells in the segmental plate.

The distinction in cell density between the rostral and caudal lateral sclerotome halves has been noted by many investigators of vertebral development in a variety of animal models (Williams, 1910; Piiper, 1928; Williams, 1942; Sensenig, 1949; Williams, 1959; Flint, 1977; Dalgleish, 1985; Verbout, 1985). The caudal sclerotome region has always been observed as a more dense accumulation of cells than the

rostral area. This difference was observed to occur after the initial motor axon outgrowths (Keynes and Stern, 1984). The possibility that the two systems are interactive, each directing the segmentation of the other in succession, has been postulated by Stern and Keynes (1987).

3. Theories of Vertebral Formation

The primary area of controversy in regard to vertebral formation has been the somitic origin of the cells which form one definitive vertebral unit (i.e. a vertebra and the caudal intervertebral disc). Many investigators have concluded that each vertebra is formed from cells of two consecutive somite pairs (Piiper, 1928; Williams, 1942; Sensenig, 1949; Williams, 1959); some researchers have contended that a vertebral unit is derived from only one pair of somites and a portion of the perichordal tube (Baur, 1969: cited in Verbout, 1976; Dalgleish, 1985; Verbout, 1985). Additionally, among the former group there is considerable difference of opinion as to the extent of contribution of each somite to each vertebra (i.e. where the original somite boundaries occur), and the position of the intervertebral disc within the single somite derivatives. This has led to further discrepancy over the fate of the rostral and caudal sclerotome halves, and over the existence of physical boundaries between these regions. Based upon independent examination of embryonic material, each investigator has formulated his/her own theory regarding somitic contribution to vertebral formation, reviewed by Verbout (1976).

From among numerous studies several have been selected for review, the selection being based upon certain criteria: the animal model used

(avian development being relevant to this study); acceptability among current embryologists (some studies have been cited more often than others); the methodologies employed; and recency of the investigation. The information gathered has been divided into sections to distinguish between the different theories, and among the methods used.

A. The Resegmentation Theory

The proponents of this theory have stated that from the initial segmental arrangement of the somites (and so of the early sclerotomes) there is a secondary segmentation that develops one-half segment out of phase with the original pattern (Remak, 1855: cited in Verbout, 1976). The initial segmental arrangement is determined by the presence of the intersomitic clefts which many investigators observed to persist following sclerotome dispersal (Piiper, 1928; Williams, 1942; Sensenig, 1949; Williams, 1959). The secondary segmentation is produced by the appearance of intrasclerotomic fissures which form approximately in the middle of each sclerotome and physically divide the rostral and caudal sclerotome halves. The intrasclerotomic fissure has been observed by numerous investigators since the first report of its existence by Von Ebner (1889: cited in Verbout, 1976): Piiper (1928), Williams (1942), Sensenig (1949), Stern and Keynes (1986), among others. The disappearance of the intersomitic cleft indicates the contacting of adjacent portions of two consecutive sclerotomes. The intrasclerotomic fissure has been traced to the intervertebral disc position and so apparently demarcates the boundary of a single vertebral unit. The location of the fissure led to the conclusion that the rostral and

caudal sclerotome halves are accordingly separated, and that each vertebra would form from the caudal halves of one pair of sclerotomes and the rostral halves of the next caudal pair of sclerotomes.

Additional evidence in support of resegmentation from morphological investigations has been generally circumstantial. It has been assumed that the sclerotome remains in the same position relative to the intersomitic vessels and to the spinal nerves. The vessels initially occupy the intersomitic clefts, but in the mature animal the segmental arteries are located at the mid-centrum level. Similarly, the spinal nerves which initially grow out through the rostral portion of the sclerotomes, in the adult can be seen to occupy positions adjacent to the caudal aspect of each vertebra. The transitory nature of many blood vessels in the early embryo is routinely included in descriptions of the formation of the circulatory system (Sadler, 1985). As well, potential shifting of spinal nerve positions resulting from a possible differential in craniocaudal growth rates of the spinal cord and mesenchymal vertebral column has not been considered.

Another of the arguments in support of resegmentation involves the formation of a functionally sound articular system. It has been claimed that the boundary shift of the vertebrae is necessary so that the vertebral muscles (derived from the myotomes) would attach across the joint (intervertebral disc) rather than having both the proximal and distal attachments to the same vertebra: a situation which would then preclude any capacity for movement. The possibility that the myotomes may shift position during development has apparently been discounted. Additionally, the supporters of resegmentation failed to

note the actual attachments of muscle to the vertebrae. There are very few muscles which attach to the middle of the centrum, most of them being attached to the neural arch and its processes: the neural arch is joined to the superior border of the centrum in most cases.

The assumptions made in the majority of these studies were necessary due to the methodologies employed: examination of serially sectioned staged embryos. These investigators were unable to identify with certainty the cells from a single sclerotome and so conclusions were drawn that were highly subject to observer bias. If one were to accept the migratory nature of sclerotome cells, in fact, it would be difficult to dispute the possibility that these cells disperse themselves randomly throughout the developing vertebral column.

B. Opposition to Resegmentation

Arguments against the resegmentation theory have generally denied the existence of the intrasclerotomic fissure (Dalglish, 1985; Verbout, 1985). It has been claimed that the presence of these fissures in longitudinal sections may be artifactual resulting from shrinkage during histological preparation (Flint, 1977). Others have suggested that a densely packed cell region (caudal sclerotome half) abutting a region of more loosely arranged cells (rostral sclerotome half) creates the impression of a fissure between the two zones (Baur, 1969: cited in Verbout, 1976; Dalglish, 1985).

The opponents of resegmentation have focused a great deal of attention on the development of the perichordal tube (Dalglish, 1985; Verbout, 1985). The cells which contribute to the perichordal tube

were, according to Dalgleish (1985), probably derived from sclerotome cells. From this point of his description he seemed to consider the perichordal tube and the remaining portions of the sclerotome (the latter of which he referred to as "the sclerotome") as entirely separate entities. Clarification of terminology would greatly facilitate communication in this field of study.

In addition to rejection of the existence of the intrasclerotomic fissures, Verbout (1985) observed that resegmentation was not necessary for the functional attachment of muscles. His schematic drawings depicted muscles attaching to the borders of the neural arches rather than to the centra. In the same series of illustrations, however, Verbout (1985) depicted the segmental vessels at the mid-centrum level, and spinal nerves at approximately the intervertebral disc level, the somite stage of development having been omitted from the sequence. In regard to the perichordal tube, he stated that he considered it "... highly probable that most of the mesenchyme in the axial area originates from the somites, [although] it is possible that it is not totally derived from the somitic mesoderm" (Verbout, 1985, 64). Furthermore, Verbout (1985) suggested that the mesenchyme which formed the perichordal tube may have been derived from angiogenic tissue.

C. Experimental Evidence

Studies which utilized somite transplants in chick-quail chimeras to study other systems have made passing remarks regarding the observation of quail cells in the vertebrae. The observations, however, have been contradictory. Beresford (1983) performed single

somite transplants in studying limb musculature formation, and noted that quail cells were present in quadrants of two adjacent vertebrae. Following double grafts of somites 6 and 7 together, however, Noden (1983) found quail cells in only C2 and C3, although labelled muscle cells were present as far caudally as the fourth cervical vertebra. Additionally, after grafting single rostral or caudal somite halves, Stern and Keynes (1987) observed that quail cells were present in either one or two vertebrae. None of these studies made any reference to the specific vertebral elements or processes which were involved, or to the extent of the involvement.

The possible clonal origin of vertebral quadrants was studied in allophenic mice by Moore and Mintz (1972). Comparisons were made of the vertebrae from tetraparental mice with normal vertebrae from each of the two purebred strains. Half-vertebrae (left or right) could usually be identified as specific to one or the other strain of mouse, although some were classified as indeterminate. Left-right asymmetry was observed in several vertebrae; occurrence of consecutive same-strain left or right vertebral halves was variable. The authors concluded that at least two clonal cell populations must have formed each vertebral half. Moore and Mintz (1972) seemed to have assumed dual somitic origin of a vertebra, and offered their findings as further evidence supporting this theory. The occurrence of vertebrae of indeterminate strain was explained by the possible mixing of cells from adjacent rostral and caudal sclerotome halves and from contralateral left and right sides. Stern and Keynes (1987), however,

have shown that the two ipsilateral populations of sclerotome cells do not mix with each other.

D. Related Evidence

In examining cell polarity in the developing vertebral column, Trelstad (1977) observed that the cells in the perichordal region exhibited a consistent pattern. At stage 27 (H & H) approximately 75% of the cells in the rostral half of each centrum were oriented with the Golgi poles directed rostrally, a similar pattern having been observed in cells of the caudal somite halves at stage 11 (H & H). The converse relationship also existed between cells of the caudal centrum halves and cells of the rostral somite halves. Trelstad (1977) noted, however, that no particular pattern of cell orientation was discernable in the mesenchymal sclerotome at stage 16 (H & H): i.e. between the somite stage and the chondrifying centrum stage. The previously mentioned relationships, then, although interesting, do not provide any evidence of cell lineage. Additionally, the cells in the developing centra were descendants of the somitic cells, not the same cells. Holtzer and Mayne (1973) have suggested that there may be several mitotic divisions between somitic cells and chondroblasts in the developing vertebral column.

Examination of vertebral formation in certain reptiles has provided indirect support for the resegmentation theory. Werner (1971) traced the intersomitic cleft to the fracture plane of autotomous tail vertebrae in lizards. The location of the split, however, has been shown to be variable among different reptiles. Some authors have

suggested that the splits developed during ossification stages and were unrelated to embryological features (discussed in Verbout, 1985).

4. Chick-Quail Chimeras

A. Development and Application of the Model

The ability to distinguish japanese quail (*Coturnix coturnix japonica*) cells from chick (*Gallus domesticus*) cells was first noted by Le Douarin (1973). Following staining with the Feulgen technique, the quail cells demonstrated large amounts of heterochromatin associated with the nucleoli, whereas in the chick cells, the DNA was generally dispersed throughout the nuclei and did not exhibit any significant nucleolar clumping. The distinction between the two cell types has been shown to occur in all cell types and throughout all stages of development (Le Douarin, 1973, 1985). This finding has provided an ideal permanent cell marker system with the formation of chick-quail composite embryos, or chimeras.

Creation of a chimera involves replacing tissue from one species with that from the other species. The usual direction of transplantation has been from quail embryos into chick embryos as a small number of quail cells are more easily located among a large group of chick cells rather than the reverse (Le Douarin, 1985). Chick-quail chimeras have been used in numerous investigations of cell migratory pathways and developmental fates in the study of various embryonic tissues and systems (reviewed in Le Douarin and McLaren, 1984). Previous examinations that followed cellular development utilized

tritiated thymidine which, due to dilution of the label with rapid cell proliferation, proved to be unreliable in many instances (Le Douarin, 1973). Additionally, the ability to distinguish between the two cell types has been used for *in vitro* studies of induction (Swalla and Solursh, 1984), cell determination (Wachtler *et al.*, 1982), and cell adhesive properties (Sanders, 1986b).

The discovery of this technique has greatly facilitated embryological investigations of cell lineage and of cell-cell interactions. Despite the fact that the embryos develop at different rates and also differ in size, in particular at hatching, some systems seem to be sufficiently compatible as to permit tissue exchanges and subsequent examination. The potential alterations in development due to the presence of heterospecific tissues, however, have received limited attention.

Kinutani and Le Douarin (1985) reported a 7.5% hatchability rate in neural tube chimeras, and of those that hatched, 37% exhibited severe abnormalities. The normal chimeras that hatched developed neurological dysfunction during the second month, possibly caused by immunological incompatibility of the tissues (Kinutani *et al.*, 1986). There would appear to be some problems associated with the use of the chick-quail chimera, although application of this model would seem to be viable within the embryonic and early post-hatching period (Kinutani and Le Douarin, 1985).

B. Properties of Chick and Quail Somitic Cells

Quail cells have been shown to be significantly more adhesive than chick cells in culture (Sanders, 1986b). Sanders (1986b) also observed a certain amount of species cell-sorting in co-cultures: in particular, quail cells tended to be located more centrally in mixed aggregates, and were absent from the peripheral regions. Some behavioral differences between the two cell species have also been observed in certain embryonic tissues (Bellairs *et al.*, 1981).

Questions have been raised as to the possible effects of the differing rates of development of the two embryos. Chick embryos hatch in approximately 21 days, whereas quail embryos hatch in 16 days. In studying somitic contribution to limb muscles, Chevallier *et al.* (1977) suggested that the "...faster differentiating quail somitic cells [may] prematurely occupy the available space" (Chevallier *et al.*, 1977, 424). The results of reverse experiments (chick somites into quail hosts) proved to be inconclusive. Very few investigations of chick-quail chimera systems have reported findings using quail as the host embryo. The difficulty of performing *in ovo* procedures in the much smaller quail egg may be one of the deterrents, in addition to the problems associated with cell identification mentioned previously.

Following implantation of a piece of quail somite into a chick embryo between the neural tube and chick somite, Erickson *et al.* (1980) noted that the implanted cells integrated with the host chick somite cells. After 48 hours almost all of the somite was comprised of quail cells. Cells from a piece of labelled chick somite similarly introduced into a host also mixed with the host cells, but after 48

hours they populated only part of the somite. It was possible that different mitotic rates of the two cell types may have accounted for the different observations.

5. Investigations of Somite Removal or Replacement

A. Somite Removal

The regulative ability of somites and the resulting effects of somite extirpation on vertebral development have been examined in various animals. The results of these studies, however, have been somewhat contradictory. Detwiler (1934) noted that in amphibians the removal of 4 consecutive somites produced the formation of irregular neural arches. In a later study, however, removal of either the medial or lateral three-fourths of the somite resulted in normal neural arches (Holtzer and Detwiler, 1953).

Working with chick embryos, Strudel (1966) claimed that when the endoderm was left intact, removal of up to 5 cervical somites resulted in no vertebral abnormalities. If, however, the endoderm was removed as well as ectoderm and somites, vertebral abnormalities were always present. Strudel (1966) concluded that intact endoderm was required in order that complete regulation occur. It should be noted that integrity of the endoderm following somite removal is difficult to determine. Similar results were obtained by Kieny *et al.* (1972) and by Menkes and Sandor (1977) in removing segmental plate mesoderm, although these investigators observed vertebral anomalies in 33% and 25% of cases, respectively. Stern and Keynes (1987) noted that following

removal of one or one-half somite, the resulting space had closed within 2 to 3 hours. The effects on morphogenesis were not included.

The findings from a recent investigation have suggested that somitic regulation may be more complex than previous results had indicated. Following single somite removals in chick embryos, Bagnall *et al.* (1986) observed normal vertebral formation in only 7 of 28 survivors after 9 days (stage 35, H & H). The remaining embryos displayed a wide range of significant abnormalities, including hemivertebrae, fusion of neural arches, and fusion of centra. The authors stated, however, that they were unable to claim 100% efficiency in somite removal with no damage to adjacent tissues.

Reproducibility of results following somite extirpation would seem to be largely dependent upon completeness of somite removal. Although Strudel (1966) claimed that he was able to remove all of the somitic cells, Kieny *et al.* (1972) stated that in their experiments total removal of the entire somite was not possible without damaging the underlying dorsal aorta. Other authors have also acknowledged that following somite or segmental plate excision some fragments of tissue may have inadvertently been left behind: in particular the mesoderm ventral to the neural tube (Menkes and Sandor, 1977; Noden, 1983; Stern and Keynes, 1987). This region of tissue cannot be visualized in a dorsal approach to the embryo, so complete removal of tissue adjacent to the notochord can be only estimated. The amount of remnant somitic tissue following extirpation may be a factor in regard to disruption of normal vertebral morphogenesis.

B. Somite Replacement

A number of investigators have made use of somite transplant procedures in studying various systems. Hazelton (1970) investigated formation of hypoglossal musculature by orthotopic grafts of tritiated thymidine-labelled occipital somites in chick embryos. In each case, somites 2 to 5 with overlying ectoderm were replaced. Hazelton (1970) reported a survival rate of 13% for embryos which were fixed from between 15 minutes and 5 days following the transplant. Notable among the results were the observations that labelled cells were found in the meninges and in the endothelium of some blood vessels.

Replacement of thoracic segmental plate in chicks by comparable tissue from another chick produced generally normal vertebral development (Kieny *et al.*, 1972). When 5 or 6 somites were replaced by other somites, however, various fusions and distortions of the vertebral elements and ribs were noted in most of the embryos at 11 days. More rib abnormalities were present in embryos which had received somite or segmental plate transplants (36 of 40) than in embryos in which the mesoderm had not been replaced (12 of 21). It may be that interactions between remnant mesoderm and implanted mesoderm resulted in the abnormalities. The cells would seem to be able to regulate to some degree in compensation for too few cells; the response to too many cells is unknown.

Noden (1983) studied the origins of craniofacial and cervical muscle using the chick-chimera model. Various combinations of transplants of somitomeres 3 to 7 and somites 1 to 7 were performed. Somites or somitomeres were always grafted in adjacent pairs together

with the overlying ectoderm. He observed that replacement of somites 6 and 7 resulted in quail chondrocytes located only in C2 and C3.

Somite contribution to intrinsic limb musculature has been extensively investigated in a number of studies (Chevallier *et al.*, 1977; Christ *et al.*, 1977; Chevallier, 1979; Beresford, 1983). Most of these investigators utilized procedures in which several consecutive somites were transferred from quail donors to chick host embryos. Beresford (1983), however, undertook a detailed mapping of the contribution of single somites to the brachial musculature. Maintenance of the correct axes of the single donor somite was claimed but not described in the paper. In addition to muscle contribution, she also observed that quail cells were found in adjacent quadrants of two consecutive vertebrae, contradictory to the findings of Noden (1983). It should be noted that Beresford (1983) grafted thoracic somites and it is possible that the level difference may account for the discrepancy in results observed.

Stern and Keynes (1987) replaced either rostral or caudal somite halves in chick embryos with corresponding tissues from quail embryos. Quail cells from each somite half were found to contribute to the same tissues: vertebral cartilage, perichondrium, intervertebral discs, ribs, musculature, and dermis. It was of note that quail cells were observed in one or two vertebrae and in one or two ribs. These authors suggested a need for re-examination of the resegmentation concept.

That the somite is regionally determined prior to segmentation has been demonstrated by Kieny *et al.* (1972). Replacement of thoracic segmental plate or somites with that from the cervical region resulted

in the absence of ribs in that region and the formation of cervical-appearing vertebral halves joined with normal thoracic counterparts on the opposite side. The converse transplant produced ribs in the cervical region and several thoracic vertebral halves attached to contralateral cervical halves. The sclerotome apparently differentiated according to its region of origin and was minimally influenced by the new local environment. The authors noted that in many instances abnormal vertebral fusions occurred, and in some cases supernumerary neural arches had formed. During transplant procedures, however, it had been noted that the implanted somites were frequently not in register with those of the contralateral side. Intraregional determination of the same tissue has not been demonstrated.

Detwiler (1938) replaced somites from *Ambystoma punctatum* with those from *Ambystoma tigrinum*. The neural arches that formed from the transplants were classified as being of donor type. The potential modifying influence of the host environment, in this case, was minimal. Based on the results of Detwiler (1938) and of Moore and Mintz (1972) it would appear that genetic limitations may direct vertebral morphology.

III. MATERIALS AND METHODS

1. Materials

A. Fertilized Eggs

Fertilized chick (*Gallus domesticus*) and quail (*Coturnix coturnix japonica*) eggs were obtained from the University of Alberta poultry farm. Quail eggs were incubated for 46 to 48 hours with the blunt end of the eggs uppermost to facilitate access to the embryos. Chick eggs were incubated for 42 to 44 hours on their sides to permit "windowing" of the eggs and later reincubation. All eggs were incubated at a temperature of 37 to 38°C at approximately 95% humidity to reach a developmental stage of 12 to 16 somite pairs (stages 11 to 12, H & H). Somites 12 to 16 have been implicated in the formation of the mid-cervical vertebrae and associated structures (Strudel, 1966).

B. Instruments and Equipment

Tungsten wire microneedles, sharpened by immersion in molten sodium nitrite (Fisher) were used to perform the microsurgical dissections. Micropipettes were prepared by pulling out sterilized Pasteur pipettes over a Bunsen burner flame. A pipette aperture diameter of approximately 10 to 20 μ m was used for removal of somite fragments from the host; an aperture diameter of approximately 200 μ m was needed for transfer of the donor somite. Somite removal and transfer were accomplished by application of suction through a mouthpiece connected with a piece of rubber tubing to the pipette.

Instruments used for initial preparation of the host and donor consisted of scalpel, blunt forceps, curved serrated forceps, and iridectomy scissors. All instruments were stored in 70% ethanol, air-dried, and rinsed in saline before use.

Solutions used in the experiments consisted of Pannett and Compton's saline (P & C) with antibiotics added (25ml penicillin-streptomycin solution [10,000 units, Sigma] per 1.0 liter of saline), Tyrode's solution, and calcium-magnesium free Tyrode's solution (CMF). These were warmed as needed by placement in the incubator. Instruments and P & C solution were sterilized by autoclaving; Tyrode's and CMF were sterilized by millipore filtration.

2. Methods

A. Experimental Procedures

1. Donor Preparation

Quail eggs were wiped with 70% ethanol and the blunt end of the shell removed to determine the position of the embryo. The embryos, including the area opaque, were cut away from the surrounding membranes, placed into Tyrode's saline in a disposable petri dish, and the vitelline membranes removed. Embryos were transferred into CMF and the ectoderm lateral to the somite columns of each was slit with a tungsten microneedle. The dish was covered and left at room temperature for 20 minutes. Embryos were then rinsed twice in Tyrode's solution. Using tungsten microneedles, for each embryo the ectoderm was reflected, the

neural tube and notochord were dissected out, and the somites were partially separated from each other. Somites could then later be removed singly as needed. The number of somite pairs segmented in each embryo was noted where possible.

11. Host Preparation

Chick eggs were removed individually from the incubator, candled over a light box, and the position of the embryo marked in pencil on the shell. The shell was wiped with 70% ethanol, and, using the tip of a scalpel blade, a small hole was made through the shell at the pointed end of the egg. A 23 gauge hypodermic needle was introduced through the hole and 0.5 to 1.0 ml of albumen was withdrawn. This procedure lowered the embryo sufficiently that it would not be damaged when the window was made. The hole was sealed with cellophane tape. With a scalpel blade, a square was scratched in the shell following the pencil outline made previously, and the square of shell was removed. The shell membrane was then peeled away, the location of the embryo was determined, and any nearby shell fragments removed. P & C was added as required to prevent drying.

A small amount of black ink (Speedball, Hunt; or Pelikan, Gunther Wagner; diluted 1:1 with P & C) was drawn into a syringe (25 gauge needle). The tip of the needle was introduced into the yolk just outside the area pellucida and the ink injected subendodermally so that the embryo could be visualized. The needle was withdrawn and the embryo staged by visual count of the number of somite pairs segmented.

The somite to be removed (usually one of the last 3 on the right side) was determined.

The vitelline membrane was split and pulled back from the region of the somite which had been selected for removal. The ectoderm over the designated somite was cut along three of the four boundaries with a tungsten microneedle and reflected. The one remaining attached border was usually the one overlying the neural tube. The somite was cut away from adjacent structures along all four boundaries. When possible a bent tungsten needle was used to lift the somite away from the endoderm and other ventral structures. As much of the somite as possible was removed using the needles. Remaining somite cells were removed by suction with a micropipette, although the ventromedial region was not visible. The pipette was drawn along all adjacent structures and also ventral to the neural tube in an attempt to clean the region. All efforts were made to minimize the damage to surrounding tissues.

iii. Transplantation Procedures

As the required techniques were found to be difficult to perfect, a grading system, based upon the quality of the transplant, was devised. Each embryo on which a transplant had been performed was accordingly categorized as belonging to one of three classes:

Class I: Transplants in which the donor and host embryos had the same number of somites (+2), the correctly matching somite was transplanted into the vacated somite space, and the somite was correctly oriented in all three planes.

Class II: Transplants in which the donor and host embryos had the same number of somites (+2), the correctly matching somite was transplanted into the vacated somite space, but the orientation of the somite was unknown.

Class III: Transplants in which the donor and host embryos may or may not have had the same number of somites and any available somite (number unknown) was inserted into the vacated somite space with the orientation of the somite unknown.

Following is a description of the procedures used in performing the Class I transplants. Procedures for the other two classes were basically the same with the exception of counting the donor somites, identifying the somite from the donor for transplant, and somite orientation.

One of the prepared donor quail embryos which matched the host chick embryo by stage was identified. The quail embryo then had the same number of somite pairs, +2 somite pairs, as the chick embryo. The specific somite desired in the quail was determined. A few carbon particles were applied to a designated edge of the somite to permit later orientation when inserting it into the somite space in the host embryo. The somite was then pulled away from adjacent somites, segmental plate, and endoderm. The somite was left in this manner while the somite space in the host chick was briefly re-examined to ensure that adjacent tissues had not expanded excessively and reduced the somite space.

The donor somite was drawn up by suction into a micropipette and rinsed in P & C. Some saline was dropped onto the chick embryo to provide fluid into which the somite could be placed, and the quail somite was transferred into the host egg near to the evacuated space. Correct orientation of the somite was determined by identification of the carbon-marked region. Using a blunt tungsten needle, the somite was appropriately maneuvered under the ectoderm flap and into the somite space. Some saline was added, the window sealed with adhesive tape, and the egg was returned to the incubator. The chick egg was out of the incubator for no more than 30 minutes (usually for 15 to 20 minutes).

iv. Problems Encountered

The somite-marking system was found to be less than ideal but seemed to be the best of all possible alternatives for single somite transplants. Problems consisted largely of insufficient adherence of the carbon particles to the somite and resulting loss of somite orientation during rinsing, transfer, or pushing the somite into position. Additionally, it was determined that when removed from its confining normal environment, a single isolated somite lost its characteristic cuboidal shape and became more spherical. Carbon particle markers on a spherical somite did not permit identification of specific somite surfaces (i.e. neural tube-facing surface, endoderm-facing surface, etc.) with certainty.

Other methods to orient the single somites were attempted but found to be less successful. Methylene blue particles placed along one

edge of the somite quickly dissolved in the saline and coloured the entire somite uniformly before the somite could be oriented. Particles of silver nitrate were found to be less adherent to the somite than were the carbon particles. Deformation of a particular corner of the somite was attempted but determined to be inappropriate as the integrity of the somite unit was possibly affected. Using a fine-mesh copper electron microscope grid single somites were lifted from the quail dish, rinsed in P & C, and transferred to the chick embryo with the orientation maintained. So much manouvering of the somite was then required, however, that orientation was lost. Retention of small tissue tags of intermediate mesoderm to the somite was also tried. Once the tissue was in the chick egg, the intent was to move the somite over the space using the mesoderm tag to orient. Using a microneedle, a cut was made to remove the excess tissue and the somite was then pushed into position. Cutting off the tag without causing injury to the underlying chick embryo or altering the position of the somite and so again losing orientation, however, proved to be extremely difficult. It was believed that for this operator, the carbon particle-marker system was the most useful, although the success rate was still very low.

A number of preliminary experiments were performed to examine some of the potential factors which may have affected survival rate, and to determine the best method of visualizing the host embryos. These have been summarized in Appendix A.

B. Collection of Material

1. Fixation

Each day, for a period of eight days following the transplant, some surviving embryos were selected randomly for fixation. Representation of most of the developmental stages between 12 and 36 (H & H) was then obtained. All dead or severely distorted embryos were discarded. For fixation, embryos were cut out of surrounding membranes and immersed in either P & C (for the younger embryos) or tap water (for the older embryos) to rinse off yolk and to remove adherent tissue. The older embryos were then placed immediately into Zenker's fixative. Each of the younger embryos were wet-mounted onto a ring of filter paper and the filter papers with adherent embryos were immersed into Zenker's fixative. The embryos were left in the fixative for varying amounts of time (8 to 18 hours) depending upon the developmental stage of the embryo.

Following fixation, embryos were rinsed in running tap water for 12 to 24 hr to remove excess potassium dichromate. The embryos were transferred into de-ionized water for 1 hr, 35% ethanol for 1 hr, and 70% ethanol for storage. Under a dissection microscope they were staged according to Hamburger & Hamilton (1951), examined for external abnormalities, and for larger embryos the cervical region was isolated. Any embryos with superficial abnormalities were discarded.

A total of 209 "quail-to-chick" somite transplants were performed: 6 Class I, 86 Class II, and 117 Class III. Forty-eight of the surviving embryos were considered suitable for further study and these

embryos were fixed, 47 for histology, and one for gross examination. Fourteen "chick-to-chick" somite transplants were completed, 2 (Class III) of these having survived for fixation. A summary of the experiments performed and the resulting losses/survivals has been presented in Table 1 (Results, p 64). Pertinent data concerning the 47 chimeras examined have been listed in Table 2 (p 77). In addition to the chimeras, a total of 45 chick and 15 quail embryos of various stages between 12 and 36 (H & H) were collected to serve as normal controls for comparison.

ii. Histology

Tissue processing for paraffin embedding was carried out according to the standard regimen (Appendix B), time for each step being 15 minutes to 1 hour, depending upon the size of the tissue. Pieces of tissue or whole embryos were oriented in the embedding molds for the desired plane of sectioning. The paraffin blocks with embedded embryos were stored in the refrigerator at 4°C until required for sectioning.

Serial sections were cut at 8 μ m on an AO Spencer microtome. Clean glass slides were smeared with a thin film of albumen fixative (Fisher) and the sections were floated directly on the slides which were then left on a warming tray until dry. Slides were processed for Feulgen/Schiff staining (listed in Appendix C). Cover slips were mounted with clear mounting medium (Permount, Fisher) and left to dry at room temperature.

iii. Somite Removal Efficiency

To determine effectiveness in removing single somites from chick embryos *in ovo*, 34 trials of somite removal were performed for examination of the embryos by stereomicroscope *in vitro*, or by scanning electron microscope. Following completion of normal somite removal, the shell window was enlarged and the embryo was cut out of the membrane. For stereomicroscope viewing (23 specimens), the embryo was rinsed in P & C and transferred to fresh saline. The embryo was then examined at high power (70X) from both ectodermal and endodermal surfaces for determination of completeness of somite removal and extent of damage to adjacent tissues.

Eleven embryos were prepared for examination by scanning electron microscopy (SEM). To facilitate transfer of the tissues through various solution changes during fixation, the embryos were wet-mounted in P & C onto filter paper rings. A standard procedure was used in preparation of the embryos for SEM (Appendix D). Embryos were examined on a Philips 505 SEM at 20kV and observations noted for each specimen.

iv. Whole Mount Specimens

To visualize the entire cartilaginous vertebral column of ten day embryos (stage 36, H & H), 18 chick and 12 quail embryos were processed according to the procedure of Simons and Van Horn (1971) for whole mount staining of chick embryos. The process involved staining the cartilaginous skeleton with alcian blue and then clearing the overlying soft tissues to render them transparent. Some embryos were also counterstained with alizarin red to demonstrate ossified regions. The

overall shape of the vertebrae and of the various processes could then be noted. This information was used to determine normal gross vertebral morphology in 10-day embryos, and to assess the shapes of the sectioned chimeric vertebrae.

C. Analysis of Results

i. Reliability of Quail Cell Identification

The investigator's ability to distinguish chick tissue from quail tissue following Feulgen staining was tested in a blind experiment. Slides were selected at random from variously staged control chick and quail embryos for examination using a Leitz Orthoplan microscope (25X objective with 10X oculars). The regions selected for examination were limited to axial structures and their precursors (i.e. somites, mesenchyme, muscle, cartilage, connective tissue). The actual species-type and the investigator's identification were recorded for each of 15 trials. Evaluation of responses was not given until completion of the entire series of slides.

ii. Examination of Sections

Viewing of slides and photomicrography were done on a Leitz Orthoplan photomicroscope. Sections of each of the experimental embryos were examined initially for quail cell content. One of the 47 embryos was found to contain no quail cells. For each of the older chimeras (21) the location of the quail cells was determined with respect to the vertebral level. Vertebrae were counted caudally from

C1 when it was identifiable, or rostrally from T1 (the first rib-bearing vertebra).

Evaluation of normal development of the quail somite was based on the assumption that quail cell content should be equivalent to one complete segment (in the younger embryos) or one complete cartilaginous vertebra and disc (in the older embryos), regardless of the distribution of the cells. It was anticipated that a range of quail cell integration into the host tissues would be observed. Embryos which exhibited the greatest content of quail cells within a given stage, and were morphologically "normal" in appearance were used as the standards to which others were compared.

In the younger embryos (25), the position of the quail cells was determined relative to the location of axial structures (notochord, neural tube). Morphological features which were present within the quail cell region (tissue differentiation, fissures, cell density patterns) were noted and progressive changes were followed through successive stages. Dispersal patterns were evaluated by comparing the quail cell positions of the various somitic derivatives (dermatome, myotome, and sclerotome).

Older embryos (21) were examined first to identify the tissues in which quail cells were present. The relative dispersal of quail derived tissues was evaluated based upon the amount of mixing of chick and quail cells. The focus of study concerned determination of quail cell content in the vertebrae. Through examination of each of the serial sections, quail cell involvement was noted as being:

- a. Extensive - many quail cells present forming a significant

- portion of the vertebral element;
- b. Moderate - a few quail cells present, but confluent with other quail cell regions; or,
 - c. Negligible - no quail cells, or very few isolated quail cells present.

These observations were made for each of the vertebral elements (centrum, disc, pedicle, lamina, spinous process, transverse process, costal process, superior and inferior articular processes) in the involved vertebrae for each of the 21 embryos. Results were compiled for each vertebral element to determine whether or not a common pattern of quail cell distribution could be distinguished.

iii. Three-Dimensional Reconstruction

To permit a three-dimensional understanding of the vertebrae involved and the quail cell dispersion, serial reconstructions were made for 9 of the 21 more mature chimeras and both of the chick-to-chick transplant embryos. For 2 embryos photomicrographs (6.3X objective with 10X oculars) were taken of every fifth section throughout the entire region that contained quail cells in the vertebral elements. The cartilaginous vertebral "slice" was outlined for each photograph, and the region containing the quail cells demarcated within these vertebral elements. For the remaining embryos, tracings of the cartilaginous region of every fifth section were made using camera lucida techniques. An attempt was made to apply computer reconstruction techniques, but the results were unsuccessful. A

summary of these computer techniques and sample printouts have been included in Appendix E.

Employing hidden lines techniques, the section outlines were sequentially superimposed to produce composite illustrations. For embryos which had been sectioned transversely, the notochord position and constant features in the spinal cord were used as references to orient successive sections. A method of "best fit" was used in reconstructing longitudinally sectioned embryos, as no constant features were present throughout the extent of the region required. Separated views were also compiled for each of the transversely-sectioned embryos. Each successive section was added with the reference point moved to the left or right, sections being separated by 3mm, 5mm, or 10mm. These views permitted a better appreciation for the depth of the vertebrae and so of the involved quail cell regions.

IV. RESULTS

1. Normal Cervical Vertebrae

A. Chick Vertebra

Through application of the technique of Simons and Van Horn (1971), the entire cartilaginous skeleton of chick embryos could be visualized (Figure 2). Examination of isolated cervical portions of the vertebral column provided more details of these vertebrae. Examples of the chick cervical column have been depicted in Figure 3. From these photographs it can be seen that the first 4 vertebrae displayed individual characteristics. The middle 8 vertebrae (C5 to C12) were similar with a gradual change toward the caudal aspect. There was some cranio-caudal overlapping of vertebral elements ventrally and laterally, but dorsally, oval-shaped gaps were present between adjacent neural arches.

The spinous processes seemed to be thickenings of the junction of the two laminae. Dorsal extension of these processes was minimal. The articular processes projected laterally from the neural arch. The facet of the inferior articular process was oriented ventrally and that of the superior process dorsally so that the joint space was approximately in a coronal plane. The column of the superior articular processes was positioned more ventrally than the inferior column: the dorsoventral distance between the two columns increased in successively more caudal vertebrae with a corresponding change in angulation of the joint space.

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FOR MICROFILMING.

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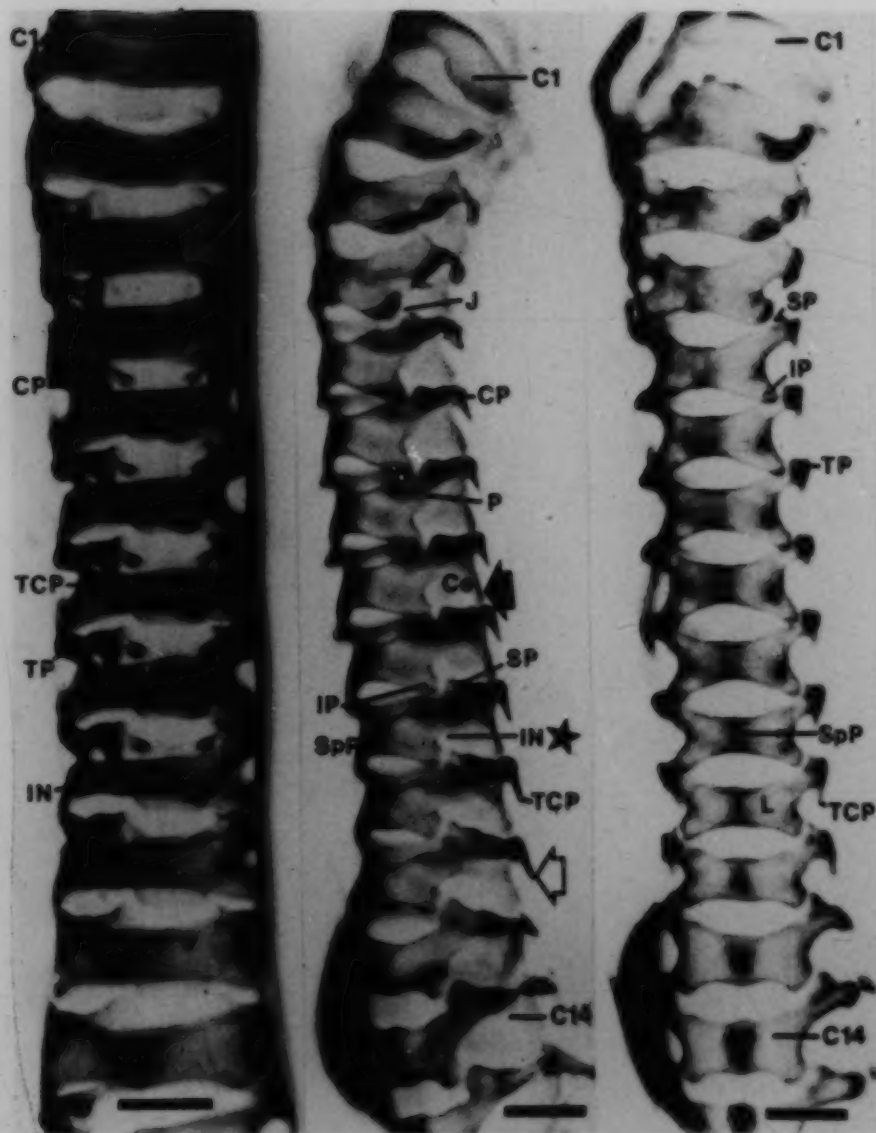
Figure 2. Cartilaginous skeleton of a 10-day chick embryo

The cartilage has been stained blue and the overlying soft tissue rendered transparent to demonstrate the chondrified skeleton.
Bar - 10mm

2

Figure 3. Views of a cervical vertebral column from a 10-day chick embryo

Ventral (left), right lateral (center), and dorsal (right) views of the cervical vertebral column. From the lateral view, a slight overlapping of the centra (Ce, solid arrow) is apparent. Dorsally, the gap between successive laminae and spinous processes can be seen. Note the inferiorly projecting tip of the costal process (TCP, open arrow) and the inferior notch of the pedicle (IN, *). Bar = 1mm



The transverse processes extended further laterally than did the articular processes and were fused to the rostral portion of the neural arch. The costal processes were located in approximately the same horizontal plane as the transverse processes, but each also possessed a caudally projecting extension on the ventral surface. Each costal process was fused to the centrum and to the transverse process forming the transverse foramen.

The two pedicles were fused to the centrum and to the rest of the neural arch. Each pedicle had a caudal notch delineating an intervertebral foramen, the position of spinal nerve exit from the vertebral column. A rostral notch was not apparent in the specimens. The centrum completed the vertebral ring ventrally. It possessed a ventral lip (hemal arch rudiment) which extended caudally and slightly overlapped the next centrum. The position of the notochord could be noted as the non-alcian blue staining circle in the middle of the centrum (Figure 4).

B. Quail Vertebra

The quail cervical column was slightly more than half (approximately 0.58) the length of the chick cervical column (Figure 5). The elements of the quail vertebrae were very similar to those of the chick, but were proportionately smaller. The dorsal gap between consecutive laminae appeared to be relatively smaller in the quail column. The quail transverse processes were not as extensive as those in the chick, and the tip of the costal process did not project as far caudally in the quail vertebra as it did in the chick vertebra.

Figure 4. Transverse plane view of a chick cervical vertebra

A single chick cervical vertebra stained with alcian blue, illustrating all the distinguishable chondrified elements. Note the position of the notochord (N) in the centrum. The transverse foramen (T) is also apparent. Bar - 100 μ m

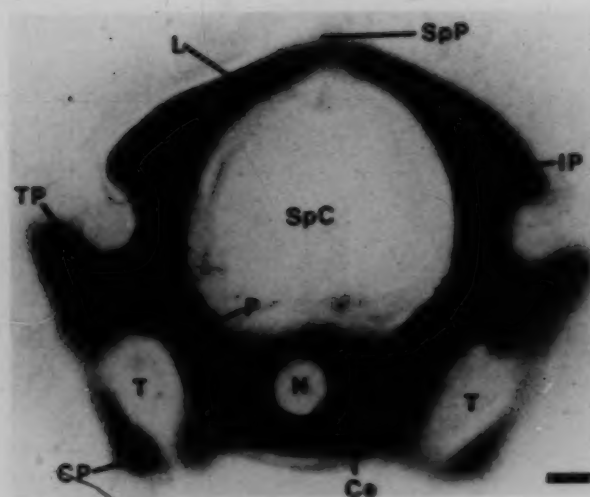
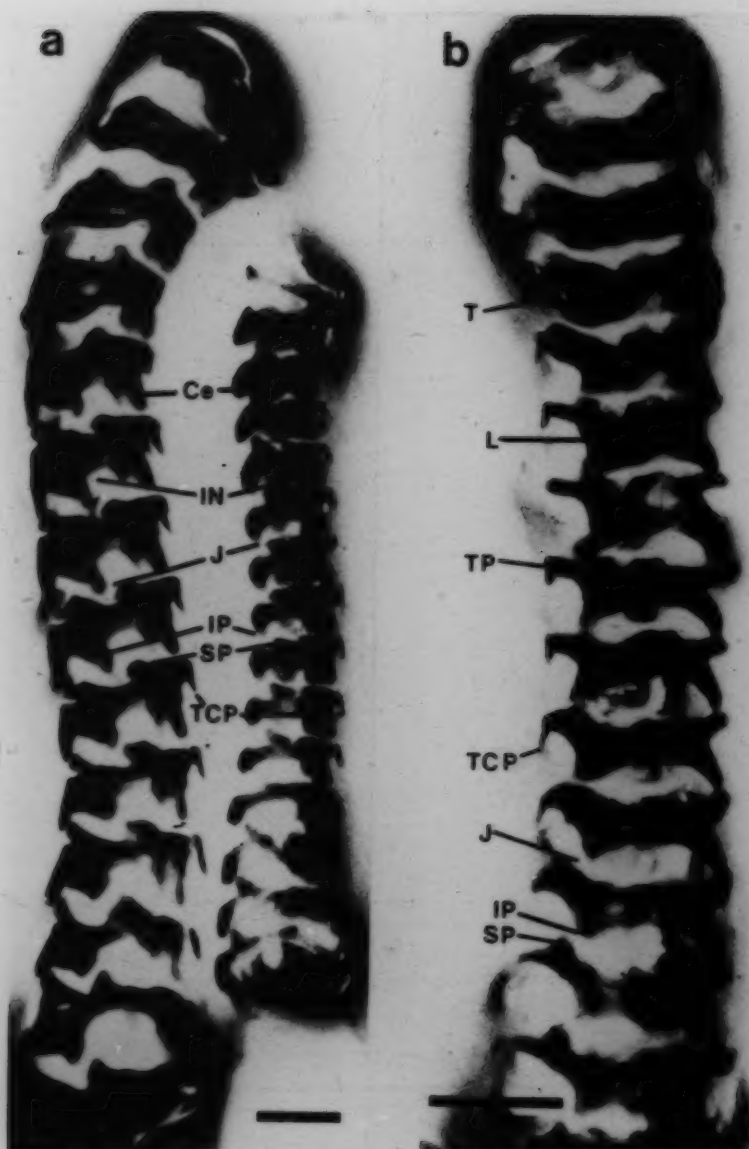


Figure 5. Views of chick and quail cervical vertebral columns

a. Ventrolateral views of 10-day chick (left) and quail (right) cervical columns. Note the disparity in size.

b. Dorsal view of a 10-day quail vertebral column enlarged.
Bar - 1mm



Differences in the shape of the spinal canal were also apparent in serial reconstructions of cervical vertebrae from the two embryos (Figure 6), with the chick spinal canal being more circular in shape than that of the quail which was somewhat triangular.

2. Results of Related Procedures

Information regarding number of survivors for each of the procedures performed has been summarized in Table 1. Operations in which neutral red had been used to visualize the host embryo resulted in a mortality rate of 76.5% after 10 days of incubation. Only 7.4% survived for the 10-day period with no detectable external abnormalities. The remaining 16.1% displayed severe abnormalities of the limbs including absence of one or more limbs, foreshortening of the limbs, and absence or malformation of one or more limb segments. Most of the older embryos that had died during incubation had similar limb defects.

For the procedures in which black drawing ink had been used to visualize the host embryo, the mortality rate was 76.1%. Among the surviving 23.9% there were a number of abnormalities noted including small eyes, lumbar spina bifida, shortened limbs, and lateral flexure of the cervical vertebral column. Several of the embryos which were sacrificed following somite transplant were also likely in a state of deterioration (notably slower heart rate) at the time of fixation which ranged from between one and 8 days following the transplant.

Figure 6. Caudal and rostral serial reconstructions of quail and chick cervical vertebrae

Serial reconstructions of a quail (right) and chick (left) C7 vertebra are presented, caudal views above, rostral views below. Costal and transverse processes are more extensive in the chick. The spinal canal is foreshortened in the quail giving a triangular shape. Bar = 100 μ m

d.

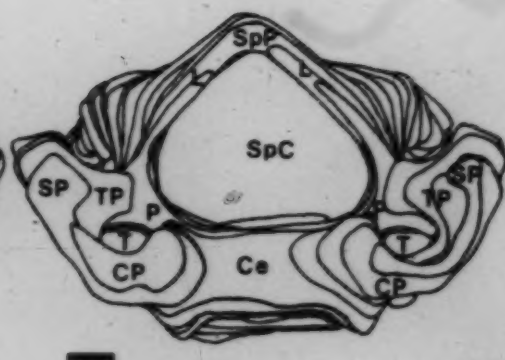
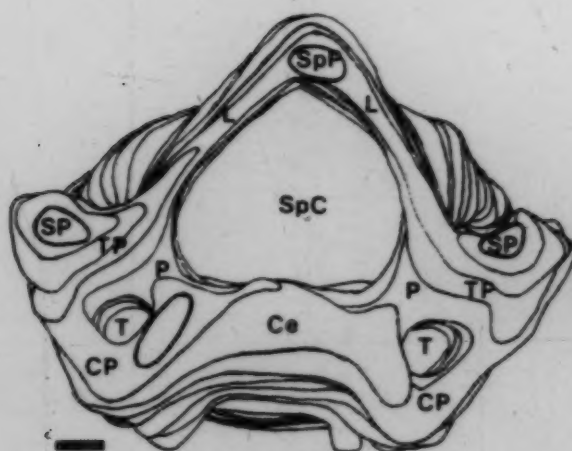
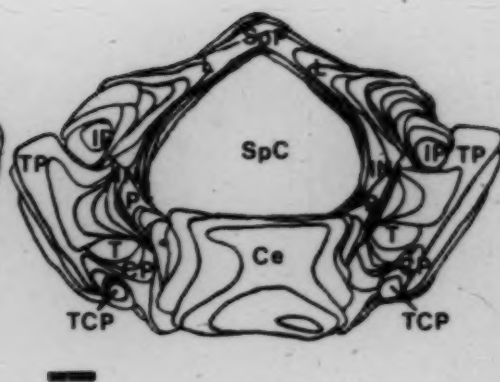
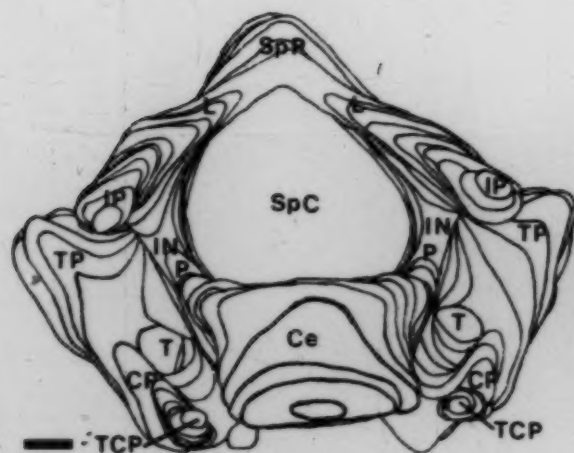


TABLE 1
SUMMARY OF EXPERIMENTS

Experiment Performed	No. Done	No. Died	No. Abnormal	No. Survived
1. Neutral red solution (0.1%) on vitelline membrane over embryos	41	27	11	3
2. Neutral red solution (less than 0.1%) on vitelline membrane over embryos	8	3	2	3
3. Agar strips with 0.1% neutral red placed on vitelline membrane over embryos	13	6	6	1
4. Neutral red solution (0.1%) on vitelline membrane which was then split	9	9	0	0
5. Using 0.1% neutral red, one somite was removed from chick embryos	44	38	3	3
6. Using 0.1% neutral red, chick to chick single-somite transplants	6	6	0	0
7. Using 0.1% neutral red, quail to chick single somite transplants	15	15	0	0
Totals of neutral red exp'ts	136	104	22	10
8. Various control experiments to determine other factors which could affect survival rate (see Appendix A)	22	5	-	17

TABLE 1 (continued)

Experiment Performed	No. Done	No. Died	No. Abnormal	No. Survived
9. Black ink in saline was injected subcutaneously to visualize the embryo	56	37	6	13
10. Using black ink, single somites were removed from chick embryos	28	25	2	1
11. Using black ink, chick to chick single-somite transplants	8	6	0	2
12. Using black ink, quail to chick single-somite transplants	209	161	-	48*
Totals of black ink exp'ts	301	229	8	64

* Of the last of these experiments, 29 survivors were of Class III and 19 survivors were of Class II (described in Methods).

A. Scanning Electron Microscopy

A surface view of the dorsum of a typical embryo from which a somite had been removed showed that damage to the ectoderm was restricted to a small square approximately the size of the somite (Figure 7a). Following removal of the ectoderm, closer examination of the site of the operation generally confirmed the observations which had been made at the time of somite removal. The results showed fairly consistent removal of most or all of the somite (Figure 7b, c). Small remnants of somitic tissue remained in the space in 6 of the 11 embryos although only 4 of these had been accounted for during the time of the procedure. It was observed, however, that the presence of somitic remnants adjacent to the notochord could not be determined during the somite removal as the overlying neural tube effectively blocked any view of this region. In 5 cases the endoderm had been torn in attempts to clean out pieces of the somite which were adhering to it. Damage to the neural tube was apparent in 3 embryos and each of those had been noted during somite removal. In 2 cases corners of one or both of the bordering somites had been nicked, these also having been reported at the time of the procedure. Somite removal was most efficient in the cases in which it was taken out as a whole unit rather than in many small pieces.

B. Feulgen Reaction

Application of the Feulgen technique to sections from variously staged control chick and quail embryos demonstrated the distinctive nucleolar marker(s) in the quail cells (Figures 8, 9). Staining in

Figure 7a. SEM of ectoderm surface following removal of a somite

A dorsal view of the embryo is shown. The cut edge of the ectoderm is indicated (*). Solid arrow marks an intersomitic cleft; open arrow marks the lateral boundary of the somite column. Bar - 100 μ m

Figure 7b. SEM of somite space following removal of ectoderm

Ectoderm has been partially removed from over the right somite column to expose the somite space. A small portion of the somite has been left behind (arrow). Bar - 100 μ m

Figure 7c. SEM of somite space following removal of ectoderm

No somitic remnants are left in the somite space. However, a few pieces of ectoderm (white flecks) are present. Bar - 100 μ m



Figure 8. Feulgen staining of 2-day control chick and quail embryos

Transverse sections through the somitic regions are shown. The nucleolar marker(s) can be noted in the quail cells (above). Chick cells (below) display a more dispersed pattern of staining within the nuclei. Bar - 100 μ m

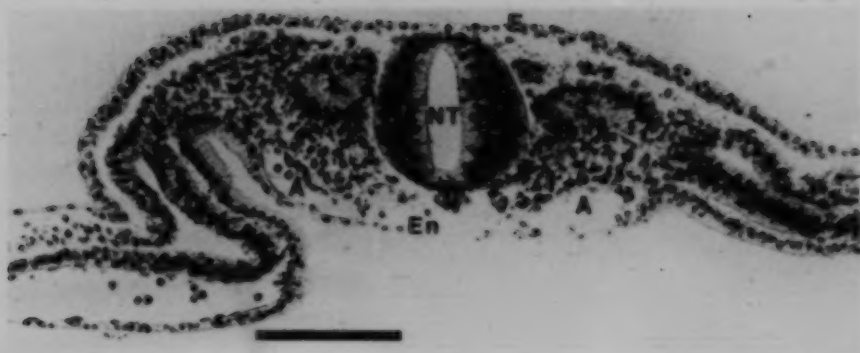
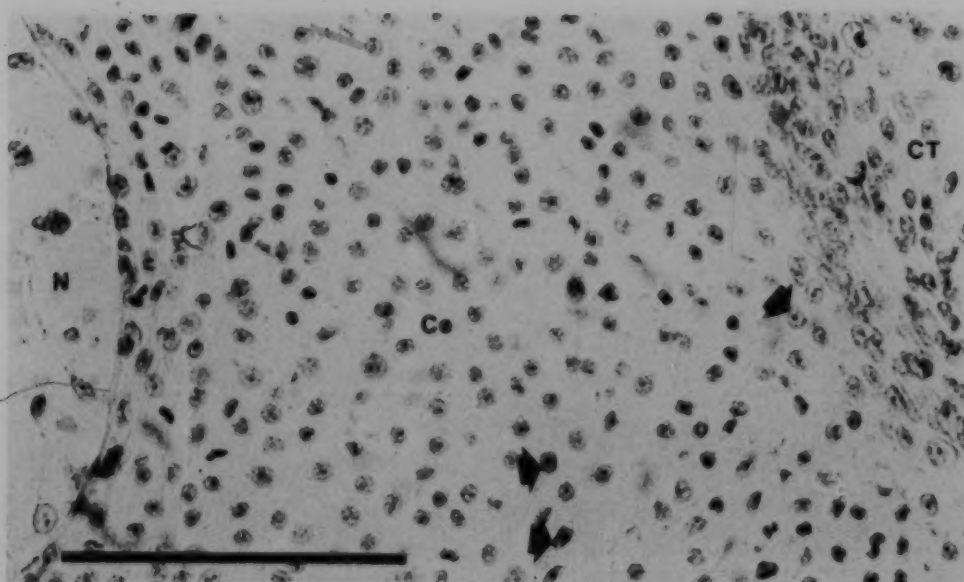
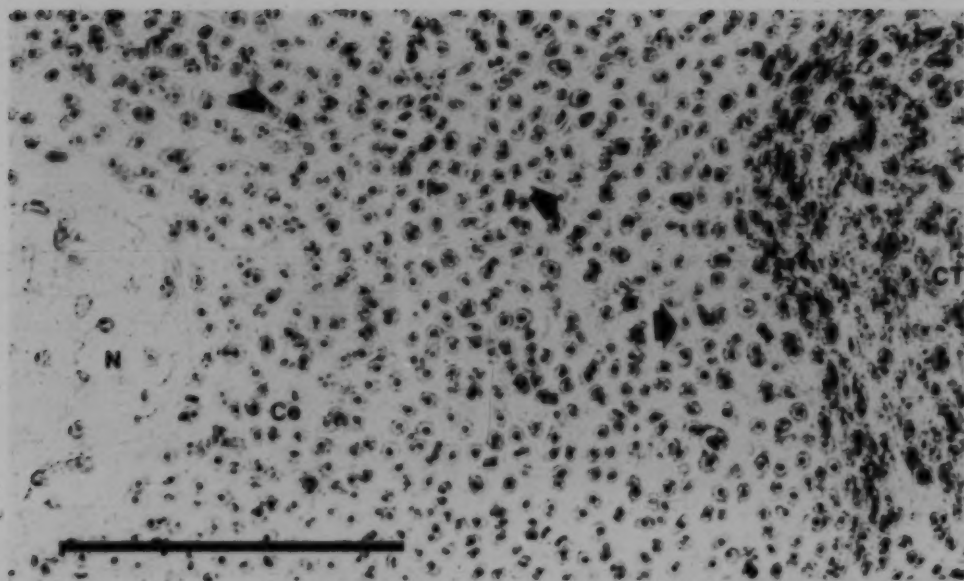


Figure 9. Feulgen staining of 10-day control chick and quail embryos

Transverse sections through the right half of the centrum cartilage of quail (above) and chick (below). Potentially non-identifiable cells are indicated with arrows. Bar = 100 μ m



the chick cells showed a more dispersed pattern of DNA (Figures 8, 9). Cells from the two species retained these staining characteristics throughout the period of time studied for all of the tissues which were examined. Within a given section, however, some isolated cells could not be classified as either chick or quail with 100% certainty. The proportion of "unknown" cell types was estimated to be 5%.

C. Identification of Quail Tissue

The investigator was able to distinguish chick and quail tissue in sections from variously staged control embryos in a blind test. In 15 of 15 trials, the investigator correctly identified the tissue as either chick or quail.

On examination of the chick tissue, some cells were observed to contain a very large, darkly stained nucleolus. These may have been necrotic cells. It was possible that these may have been mistaken for quail cells in some cases. When both nucleoli were fully within the plane of section, however, it could be noted that the quail nucleoli were distinctly smaller than those of the chick cells.

3. Chick to Chick Somite Transplants

Two of the 14 homospecific transplant embryos survived to fixation at 10 days. Embryo 27/5 had received a chick somite (level undetermined) at somite number 11 (of 12 segmented). Embryo 27/8 had received a chick somite (level undetermined) at somite number 13 (of 14 segmented). Examination of serial sections demonstrated extensive abnormalities of C5 and C6 for 27/5, and of C8 and C9 for 27/8.

somite-vertebral difference was then 5 and 4 for the two embryos, respectively. Abnormalities included unilateral fusion of the centra, absence of costal and transverse processes of C6, and atypical morphology of both of the C5 articular processes and the C6 superior articular process for 27/5. Embryo 27/8 exhibited almost total unilateral fusion of the two vertebrae. The contralateral side was also affected with the C8 pedicle displaced laterally and the two centra fused. It was notable that, for each of the two embryos, the somite which had been transplanted was from the anterior portion of the donor's somite column.

4. Quail to Chick Somite Transplants

Forty-eight chimeras were collected following replacement of single chick somites with quail somites. One embryo was allowed to develop to 21 days, but it died before hatching. Superficial examination of the embryos showed no detectable external abnormalities. Of the 47 embryos which were fixed for sectioning and Feulgen staining, 46 were shown clearly to contain quail cells. In the remaining embryo (35/2) no quail cells could be found. An example of chimeric tissue in which the distinction between chick and quail cells could be noted has been illustrated in Figure 10. Data regarding the somites involved for each of the chimeras collected have been summarized in Table 2.

In each of the 46 chimeras, the quail cells were located primarily in the axial region, extending also to sub-ectodermal positions. The quail cells generally showed minimal intermingling with the chick cells

Figure 10. Feulgen staining of mesenchyme from a stage 23
chimera

Note the distinction between quail cells (large solid arrows) and
chick cells (open arrows). A few cells of uncertain species
(small arrows) are present. Bar = 100 μ m

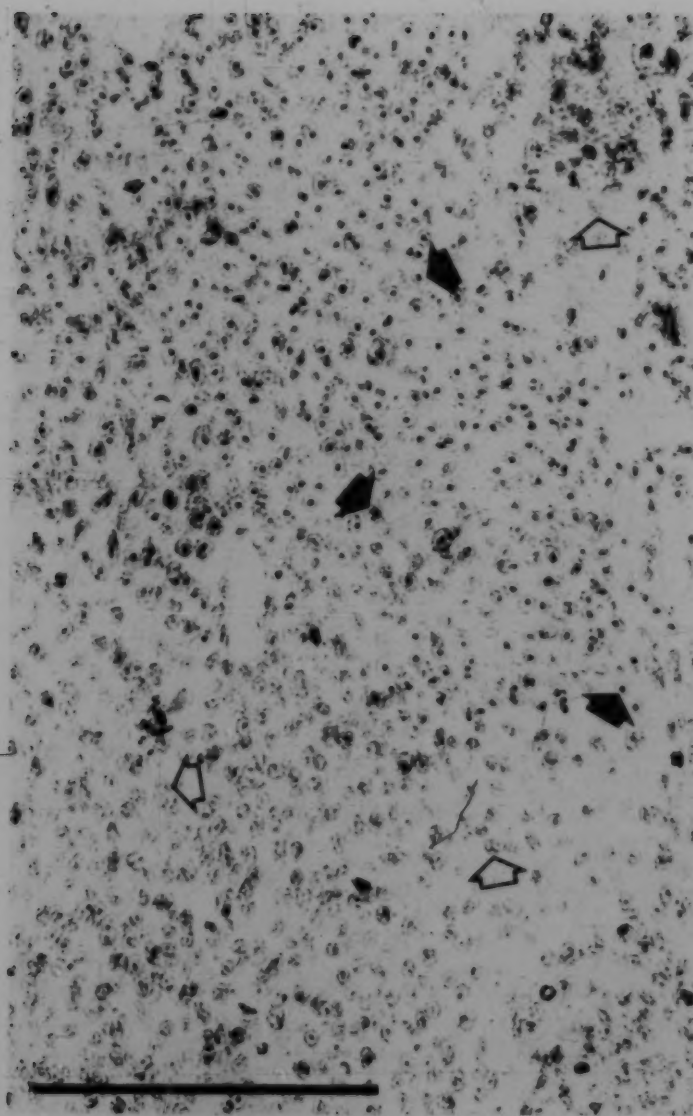


TABLE 2

DATA SUMMARY OF 47 EMBRYOS

Embryo	Host			Donor			Stage	Plane of
no.	Som rem	Tot pr	Mat	Som tra	Tot pr	Mat	at fix	section
26/5	8r	14	6	u	u	u	35	T
29/2	8r	9	1	u	u	u	17	T
29/3	8r	9	1	2r	10	8	20	S
29/4	14r	16	2	3r	10	7	24	C
29/6	13r	16	3	3r	12	9	14	T
29/7	14r	18	4	u	u	u	16	T
30/1	9r	10	1	u	9	u	15	S
31/1	13r	14	1	15r	17	2	13	T
31/2	16r	19	3	14r	17	3	28	S
31/3	15r	16	~~1~~	~~12r~~	14	2	29	C
31/4	16r	20	4	13r	17	4	28	C
33/1	10r	12	2	16r	17	1	18	T
34/4	9r	11	2	10r	11	1	29	C
35/1	7r	8	1	7r	8	1	24	T
35/2	12r	13	1	u	u	u	26	C

Som rem - number of the somite removed

Tot pr - total number of somite pairs present

Mat - maturity of somite (total somites - number of somite removed)

Som tra - number of the somite transplanted

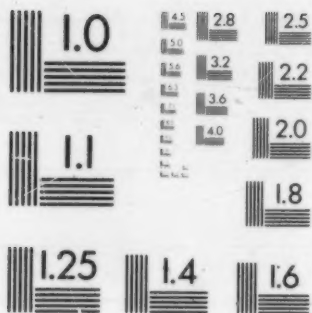
Stage at fix - stage of the embryo at fixation (H & H)

r - right

l - left

u - undetermined

2



MICROD

TABLE 2 (continued)

Embryo no.	Host			Donor			Stage at fix	Plane of section
	Som rem	Tot pr	Mat	Som tra	Tot pr	Mat		
38/2	16r	17	1	15l	16	1	28	C
38/3	16r	18	2	16l	16	0	35	T
38/4	18r	20	2	u	16	u	17	C
38/6	15r	17	2	u	14	u	36	T
39/3	9r	11	2	9r	12	3	15	T
39/4	10r	12	2	10r	12	2	14	T
40/1	11r	13	2	10r	12	2	28	C
40/2	9r	11	2	9r	11	2	12	C
40/4	9r	9	0	9r	12	3	26	C
40/5	10r	11	1	10r	12	2	13	T
42/3	12r	15	3	12r	14	2	14	C
42/4	13r	15	2	13r	14	1	13	T
42/6	13r	15	2	12r	14	2	20	T
43/1	12r	15	3	9r	10	1	25	T
44/2	16r	18	2	14l	14	0	35	C
52/2	16r	17	1	9l	13	4	30	T
52/5	15r	18	3	u	u	u	30	C
53/5	9r	10	1	9r	11	2	23	C
53/7	14l	15	1	14l	15	1	24	T
54/2	13r	18	5	9l	u	u	24	T
54/4	20r	22	2	21s	10	u	23	T

TABLE 2 (continued)

Embryo no.	Host			Donor			Stage at fix	Plane of section
	Som rem	Tot pr	Mat	Som tra	Tot pr	Mat		
55/3	16r	19	3	16r	19	3	22	C
55/5	16r	19	3	16r	19	3	22	T
75/1	16r	17	1	10l	12	2	18	C
75/3	10r	11	1	10r	12	2	36	T
83/1	15r	17	2	8l	12	4	26	T
83/3	13r	15	2	13r	13	0	27	C
92/1	16r	17	1	16r	17	1	30	C
92/3	11r	12	1	11r	13	2	28	C
95/4	18r	19	1	18r	19	1	33	S
96/2	13r	13	0	13r	13	0	31	C
96/5	15r	16	1	o	u	u	32	C

Som rem - number of the somite removed

Tot pr - total number of somite pairs present

Mat - maturity of somite (total somites - number of somite removed)

Som tra - number of the somite transplanted.

Stage at fix - stage of the embryo at fixation (H & H)

r - right

l - left

u - undetermined

o - oriented somite

2ls - 2 left somites were inserted into a single space

C - coronal

S - sagittal

T - transverse

contained in the still-intact implanted somites. With advancing maturity, an increase in number of quail cells and in the area occupied by quail cells could be noted. In older embryos, quail cells were found in a variety of tissues including muscle, cartilage, meninges of the spinal cord, and mesenchyme of presumptive dermal connective tissue and general connective tissue. For description of observations, the 46 embryos have been separated into three groups based upon maturity of the axial region.

A. Early Phase - Stages 12 to 16 (H & H)

The first group of embryos included all stages of development from the intact somite to just prior to the disappearance of the perinotochordal space in the area of interest. Ten embryos were in this group, ranging from stages 12 to 16 (H & H) inclusive. The host somites extended over a span of 6 somite levels. Within this group, 3 embryos displayed either extreme tissue necrosis, or minimal to no integration of quail cells into the host tissue. These have been excluded from the description of somite development. The 2 specimens which showed minimal or no integration had both received transplants of a quail somite of undetermined level. Of the remaining 7 embryos, 5 had received a quail somite which corresponded exactly to the chick somite removed (Class II transplant). The somite maturity difference in this group of 5 was zero or one (i.e. embryos were of matching stages as determined by somite count). This information has been summarized in Table 3.

TABLE 3
SUMMARY OF SOMITE VARIABLES FOR
EMBRYOS OF STAGES 12 TO 16

Somite No. -	Embryo Numbers		
Removed			
9	30/1	39/3	40/2
10	40/5	39/4	
12	42/3		
13	29/6	31/1	42/4
14	29/7		

Difference in Somite Levels

0 2 10 u

	0	40/2 39/4	
Difference in Somite Maturity	1	39/3 40/5 42/3 42/4	31/1
	6		29/6
	u		30/1 29/7

u - undetermined

1. Somite Development

The quail somites had generally integrated normally into the chick host as determined by comparisons with adjacent somites. In the earliest stage, it was noted that the size and shape of the quail somite approximated that of the contralateral and the immediately rostral and caudal chick somites (Figure 11). The somites in early stages were cuboidal in shape. With disruption of the somites they became more wedge-shaped, the dorsolateral surface being the base of the wedge (Figure 12). The somites also developed an oblique orientation with the extreme caudal portion of the somite situated ventromedially, and the extreme rostral aspect located dorsolaterally.

Breakdown of the somites was evident at stage 13 (H & H) and was initiated on the ventral and ventromedial surfaces of the somite. An intact epithelial layer of dermatome located dorsolaterally was distinguishable from the mesenchymal sclerotome cells (Figure 12). In many sections there was a distinct separation of these two regions resulting in the appearance of the myocoele, remnant of the somitic cavity. The sclerotome region extended from the dermatome ventrally to the ipsilateral dorsal aorta, ventromedially toward the notochord, and medially to the neural tube. The cell density of the sclerotome decreased progressively from the dorsolateral myocoele to the ventromedial regions. This progression appeared to be continuous in these stages with no demarcation between the loose-cell zone and the dense-cell zone.

Immediately surrounding the notochord a virtually cell-free space (perinotochordal space) was observed. The perinotochordal space was

Figure 11. Longitudinal sections of a stage 14 embryo (42/3) showing intersomitic clefts and intrasclerotomic fissures

Dorsal (above), middle (center), and ventral (below) regions of the quail somite. Sclerotome dispersal has begun in this embryo. Intersomitic clefts (open arrows) and intrasclerotomic fissures (solid arrows) can be seen. Bar - 100 μ m

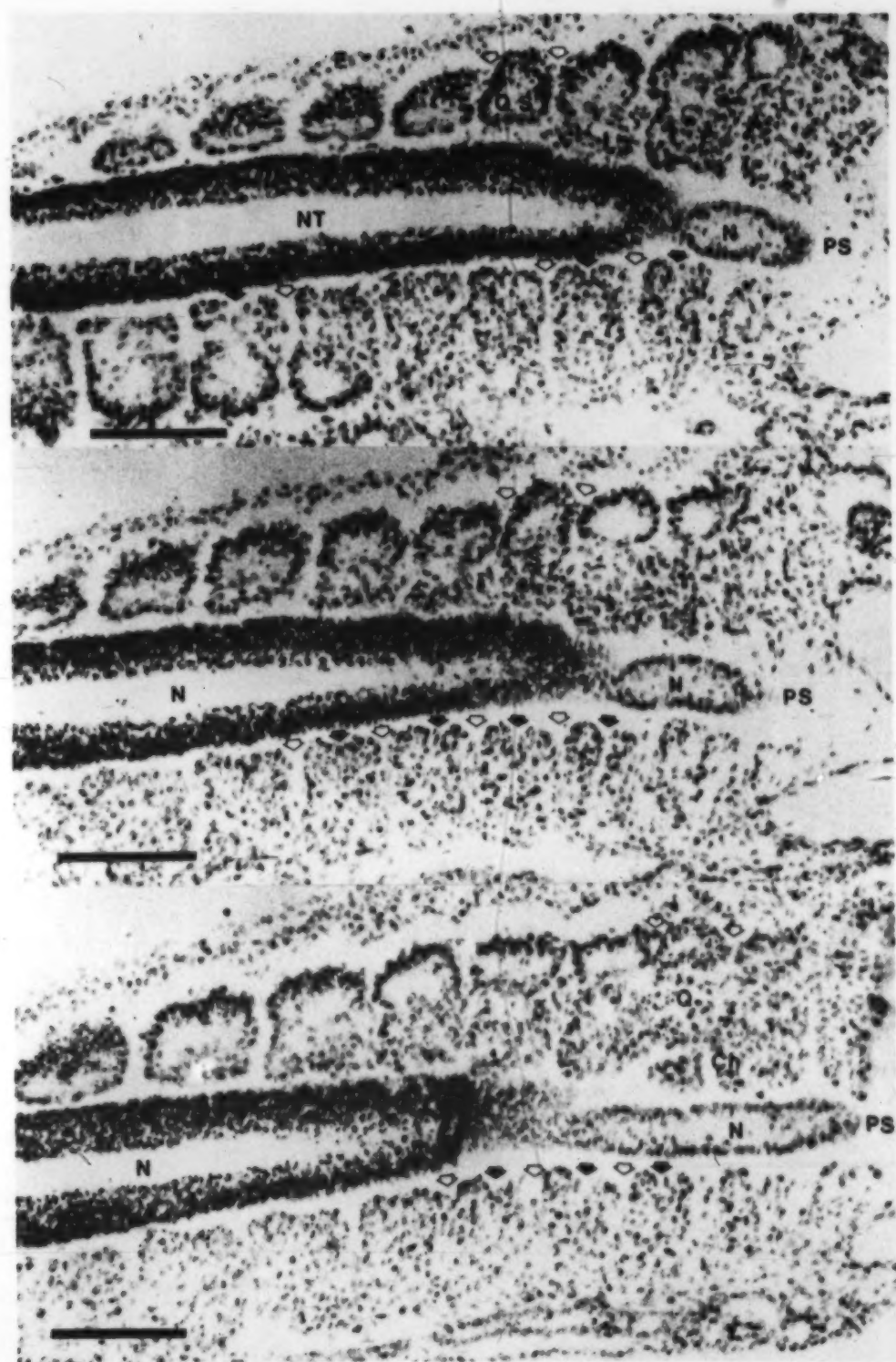
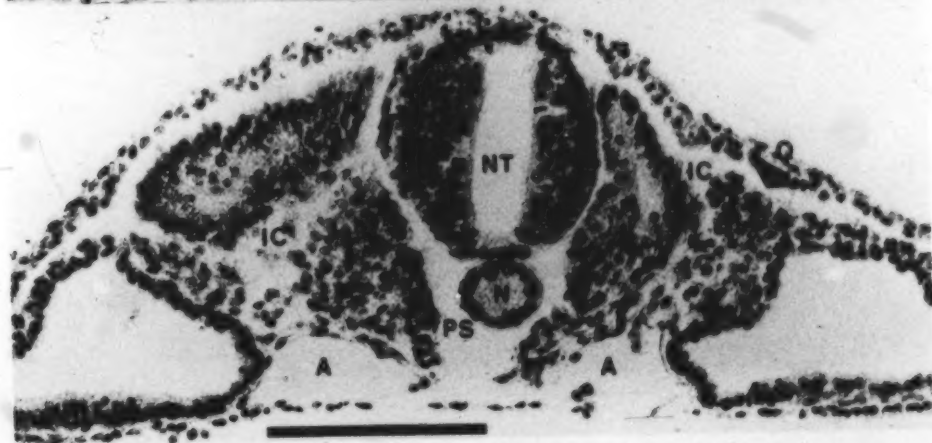
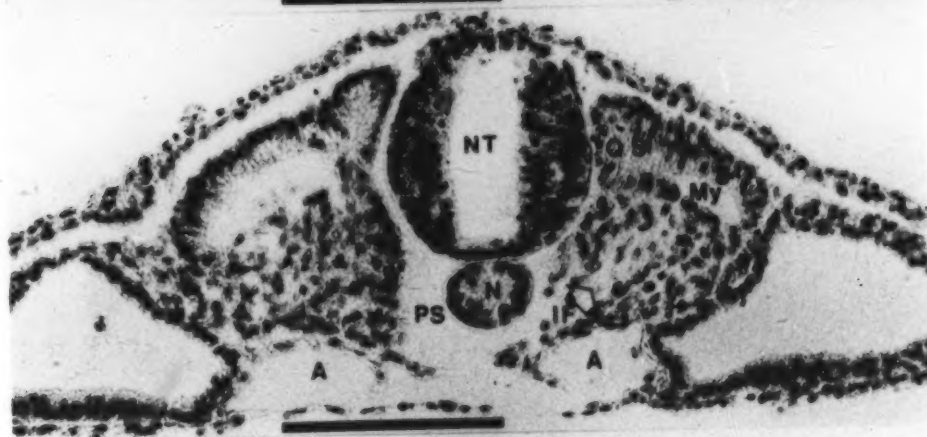
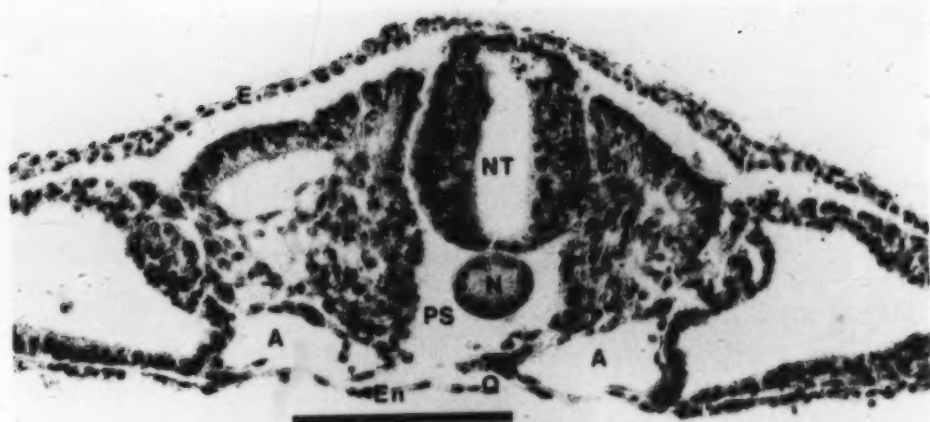


Figure 12. Transverse sections of a stage 13 embryo (40/5) showing quail sclerotome dispersal and the perinotochordal space

Caudal (above), middle (center), and rostral (below) regions of the quail somite. The perinotochordal space is less prominent on the quail (right) side. The loose-celled region within the quail sclerotome seen in the center section may be the intrasclerotomic fissure (open arrow). Intersomitic clefts are present due to the obliquity of the sclerotome and of the sections. Bar = 100 μ m



reduced progressively through successive stages, persisting until stage 17 (H & H). Sclerotome cells were not seen as a constant feature in the mid-sagittal plane until stage 14 to 15 (H & H). This event appeared to be coincident with fusion of the dorsal aortae into the definitive aorta (Figure 13). Prior to this time there existed a mid-sagittal cell-free zone between the notochord and the endoderm. No comparable perineural space was seen lateral to the neural tube. The cells of the notochord and neural tube remained in close apposition throughout these stages with no quail or chick cells intervening. By stage 15 (H & H) the quail sclerotome cells were seen to abut the contralateral chick sclerotome at the mid-sagittal plane, but no quail cells were observed which had crossed this plane to the opposite side of the embryo.

The dermatome forming the dorsolateral boundary of the somite had a distinct dorsomedial corner with a continuation extending a short distance ventrally along the neural tube (Figure 12). The dermatome was initially a single-layered and later a double-layered epithelium with formation of the dermomyotome at stage 14 (H & H) (Figures 12 and 13). With advancing age, the dermatome and myotome reached progressively further in a ventrolateral direction. Some fusion of adjacent myotomes began at stage 14 (H & H) but the junctions were still apparent as the intersomitic fissures.

In all stages of this phase of development and throughout the extent of the somite (dermatome, myotome, and sclerotome) the intersomitic clefts could be distinguished. In the earlier stages this region existed as an acellular zone (Figure 11), but in later stages it

Figure 13. Transverse sections of a stage 15 embryo (39/3) showing quail cells in the mid-sagittal plane

The dorsal aortae are fused in this embryo. Note the presence of quail sclerotome (left) cells (arrows) in the mid-sagittal plane. Bar = 100 μ m



2

contained a few cells. Some isolated quail cells were seen within the sclerotome of adjacent ipsilateral chick sclerotome but largely the quail cells remained in the region delimited by the intersomitic clefts. Intersomitic blood vessels were not observed in these gaps until later stages. In some sections of embryos at stages 13 and 14 (H & H) a loose-cell zone was noted within the sclerotome from one somite (Figure 12). This feature may be the intrasclerotomic fissure. In one embryo sectioned longitudinally, a gap could be seen within the loose sclerotome in ventral areas, and a small medial indentation noted in the wall of the intact somite located dorsally (Figure 11). The intrasclerotomic fissure was not prominent in embryos of stages 15 and 16 (H & H).

What were assumed to be chick neural crest cells were seen between the somite and neural tube (Figures 12, 13). No chick cells identifiable as neural crest could be seen within the sclerotome regions in these stages. Chick cells were seen to form all of the structures surrounding the quail somite (i.e. notochord, neural tube, aorta, ectoderm, endoderm, nephrotome, and lateral plate mesoderm). No quail cells were observed which had been integrated into these tissues, although quail cells were frequently observed in close proximity to them.

ii. Abnormalities

Although the quail somite dimensions approximated those of adjacent chick somites, some disparity was noted. Smaller rostrocaudal length of the quail somites was coincident with lateral flexure of the

embryos. The concavity faced the operated side and the center of the flexure was located at the site of the quail somite. In most embryos the mediolateral width of the quail somite region was smaller than the opposite chick side resulting in a compressed appearance of many of the sections.

Cell necrosis was very extensive in one of the 10 embryos. Deterioration of tissues seemed to be starting in 4 other embryos. Some information could be obtained from sections of these embryos although the quail cells were somewhat difficult to identify in photographs.

In 2 embryos, the implanted quail somite had not been integrated normally into the host. In one of these (29/7), the somite had remained as an epithelial ball of cells located between the ectoderm and the underlying chick somitic tissue (Figure 14a). In the other embryo (30/1), the quail somite had separated into two regions of quail cells which remained clustered together and were completely surrounded by chick cells. The first embryo (29/7) had received a very rostral quail somite (number undetermined) which could not be pushed into the plane of the somite column at the time of the transplant. Embryo 30/1 also had received a quail somite of undetermined number, and it was noted during the transplant that the somite might not have been entirely cleared of adjacent tissue. Neural tube abnormalities were evident in 2 embryos.

The dispersal of the quail somitic cells was variable among the group of 7 embryos analyzed. In one embryo (40/2) the sclerotome seemed to be dispersing in a lateral rather than a medial direction

Figure 14a. Transverse section of a stage 16 embryo (29/7) showing non-integration of the quail somite

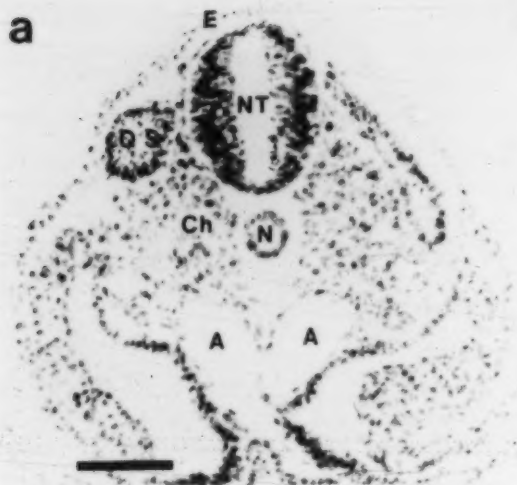
An example of non-integration of the quail somite into the chick host is presented. The quail somite remained an intact epithelial ball just deep to the ectoderm. Chick mesenchyme has filled the space between the quail somite and the axial structures. Bar = 100 μ m

Figure 14b. Longitudinal section of a stage 12 embryo (40/2) showing lateral rupture of the somite

Quail sclerotome (or mesenchyme) appears to be dispersing laterally at this level in the embryo. The quail somite is marked with an open arrow. Some chick tissue (solid arrow) is possibly present adjacent to the neural tube and within the quail region. Bar = 100 μ m

Handwritten scribble or signature.

a



b



(Figure 14b). In sections from 4 of the 7 embryos a variable amount of chick tissue was present within the quail-cell region. No indication of remnant somitic tissue had been noted at the time of the transplant. The chick cells were located in the medial (one embryo) or ventromedial (3 embryos) regions of the sclerotome and appeared to be mesenchymal cells. In 3 of the 4 cases a demarcating cell-free zone was present between regions of these two cell populations within the boundaries of the single somite (Figure 15). The groups of chick cells were similarly separated from cells of adjacent chick somites by the intersomitic fissures. In the fourth embryo (29/6) there was no distinct gap between the chick and quail cell groups within the quail somite level (Figure 15). Considerably more intermingling of the two cell types was also apparent. This embryo had received a quail somite which was 10 positions more rostral than the somite it replaced (Table 3).

iii. Summary of Early Phase

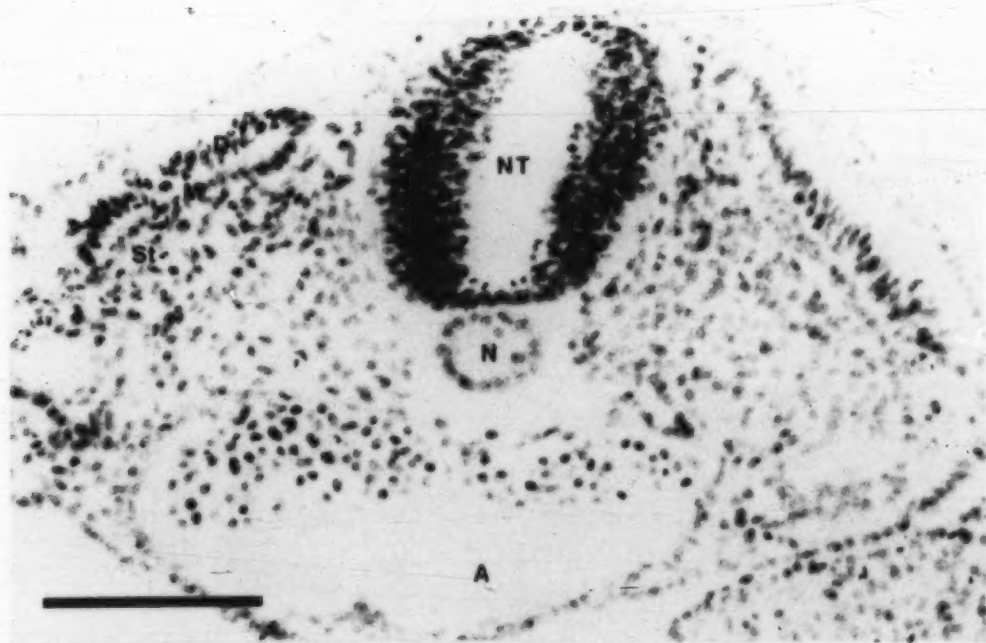
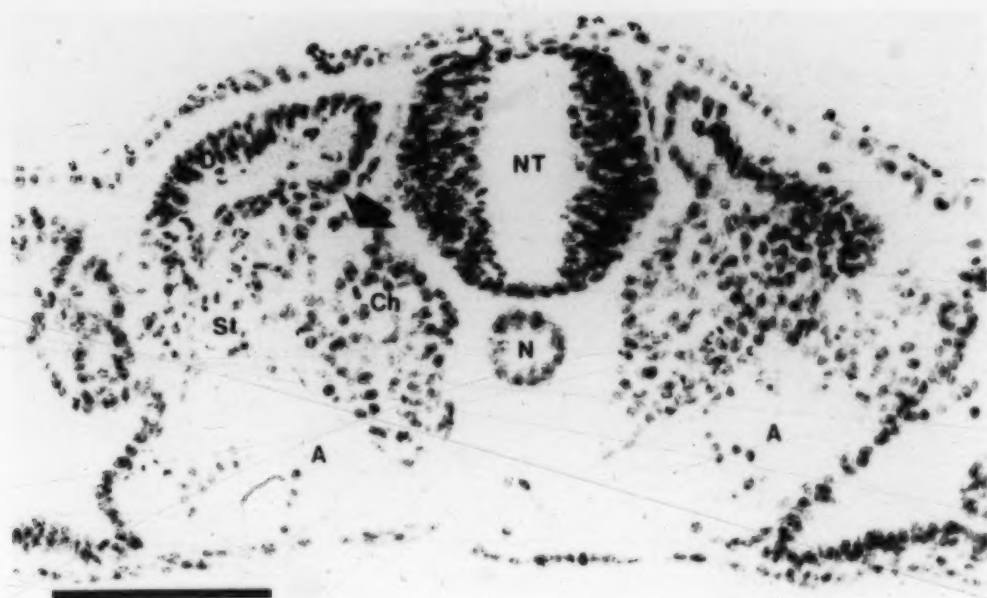
A number of features was notable during the early stages of development.

1. The quail cells were located almost entirely within a delineated region.
2. Breakdown of the ventromedial epithelium of the somite resulted in formation of distinct dermatome and sclerotome cell populations in the quail region.
3. The quail sclerotome abutted the neural tube but left a cell-free zone immediately surrounding the notochord.

Figure 15. Transverse sections of stages 13 (31/3) and 14 (29/6) embryos showing chick cells within the quail region

The ventromedial portion of the quail region contains primarily chick cells in both embryos. Formation of dermatome and myotome can be noted in the quail somite below. An apparent gap (arrow) separating chick and quail cells within a single segment is present above.

Bar - 100 μ m



4. Intersomitic clefts were prominent throughout this phase of development.
5. Intrasclerotomic fissures may have been observed but these were not pronounced features.
6. Lateral flexure toward the operated side seemed to be related to the smaller rostrocaudal dimension of the quail somite as compared to adjacent chick somites.
7. Abnormalities were associated with non-matching somites in 3 of 5 cases.

B. Intermediate Phase - Stages 17 to 25 (H & H)

The intermediate phase of development included embryos from the time at which the perinotochordal space had disappeared (stage 17, H & H, approximately 64 hours) to just prior to the appearance of cartilaginous or precartilaginous vertebral anlagen (stage 25, H & H, approximately 4 days). Fifteen embryos were included in this group. The somites removed from the host chick embryos ranged from somite 7 to somite 20. The difference in level between the somite removed and the quail somite implanted varied from zero (5 embryos) to 11 (one embryo) with 3 having been undetermined. The difference in maturity of the somites ranged from zero (4 embryos) to 7 (one embryo) with 4 undetermined. This information has been summarized in Table 4.

For 2 embryos (29/2 and 75/1) the extreme amount of tissue necrosis in the sections severely limited the amount of information that could be obtained. These have been excluded from the discussion of normal development. Among the rest of the embryos, the large amount

TABLE 4

SUMMARY OF SOMITE VARIABLES FOR
EMBRYOS OF STAGES 17 TO 25

Somite No.		Embryo Numbers					
	7	35/1					
	8	29/2	29/3				
	9	53/5					
	10	33/1					
	12	43/1					
	13	42/6	54/2				
	14	29/4	53/7				
	16	55/3	55/5	75/1			
	18	38/4					
	20	54/4					
Difference in Somite Levels							
	0	1	3	4	6	11	u
	0	35/1 53/7 55/3 55/5					
Difference	1	53/5	42/6		75/1 33/1		
in Somite	2		43/1				
Maturity	5					29/4	
	7				29/3		
	u			54/2		29/2 38/4 54/4	

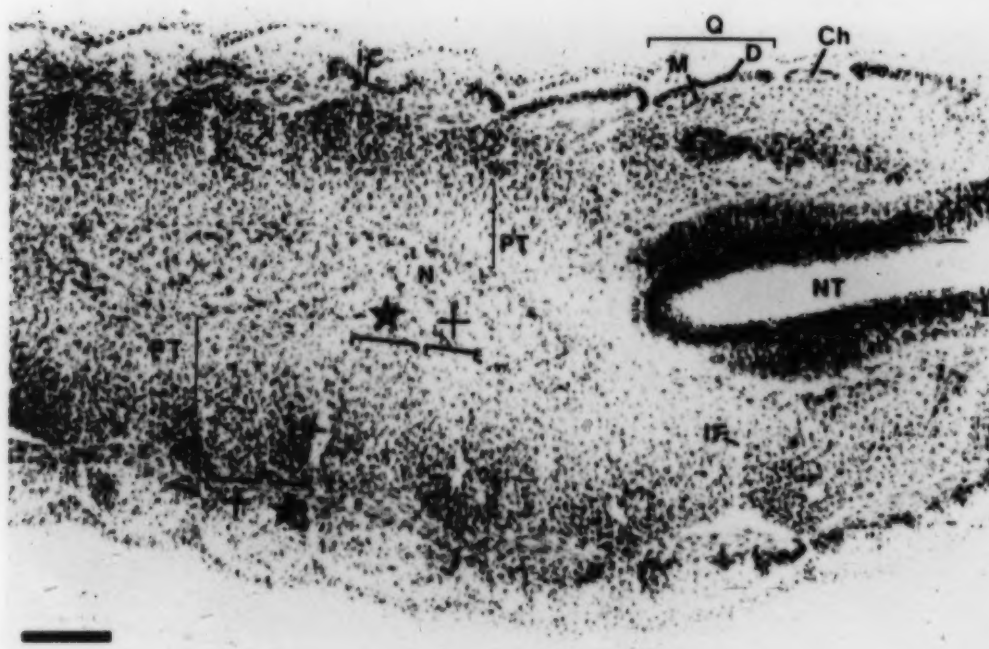
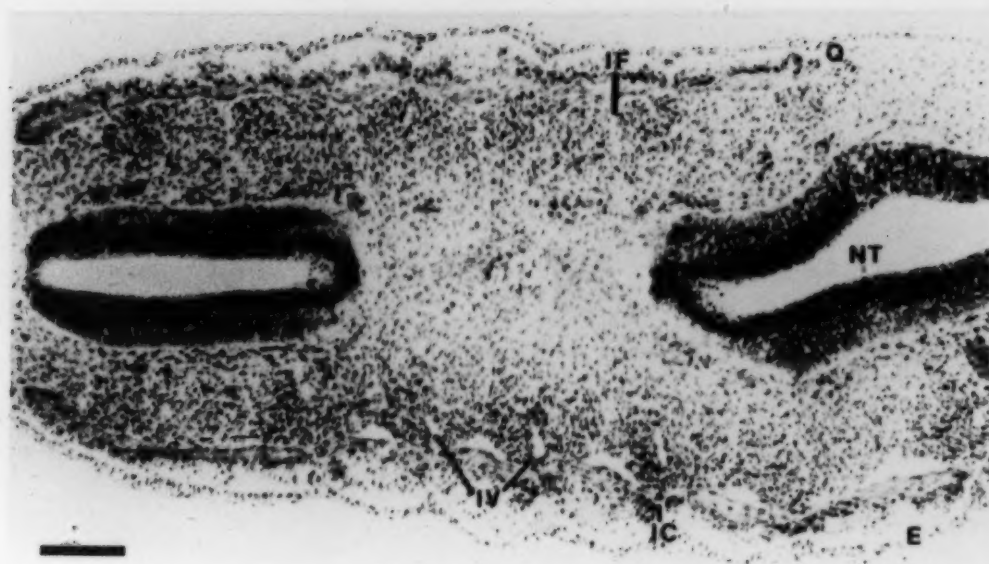
of variability in the location of the spinal ganglion and in the relationships of the various cellular condensations rendered determination of normal developmental pattern within the quail cell region extremely difficult. In some cases description has been taken from adjacent chick regions with the assumption that the somite segments were a repetitive series of similar units.

1. Somite Development

At stage 17 (H & H) the perinotochordal space was obliterated and the sclerotome cells could be seen in close association with the notochord. Immediately surrounding the notochord was a relatively loose zone of cells (perichordal tube or sheath) compared to a dense-cell zone in the lateral sclerotome (Figure 16). No discontinuity was observed between these two zones although the transition was rather abrupt. In some sections there appeared to be regions of differing cell density within the perichordal tube. The region of the intersomitic cleft was an area of more loosely associated cells, this zone seeming to continue into the caudal portion of the next rostral sclerotome. There was a gradual increase in cell density rostrally such that a dark and light staining pattern was produced (Figure 16). This pattern was not as distinct as that within the lateral sclerotome region. Laterally the intersomitic vessel separated a dense area in the caudal sclerotome half from a less dense area in the rostral sclerotome region of the next caudal segment (Figure 16). Some sections were seen in which there was an actual separation of these two zones: the intrasclerotomic fissure. This fissure was an inconstant

Figure 16. Longitudinal sections of a stage 17 embryo (38/4) showing distinction between the perichordal tube and lateral sclerotome region

Cell density patterns in the chick region are distinct in dorsal (above) and ventral (below) sections. The perichordal tube (PT) can be distinguished from the lateral sclerotome area. Dense (*) and loose (+) cell zones are present in each. Intersomitic clefts (IC) and intrasclerotomic fissures (IF) can be noted.
Bar - 100 μ m



feature, but when present it was always located directly opposite the apex of the myotome convexity of that segment (Figure 16).

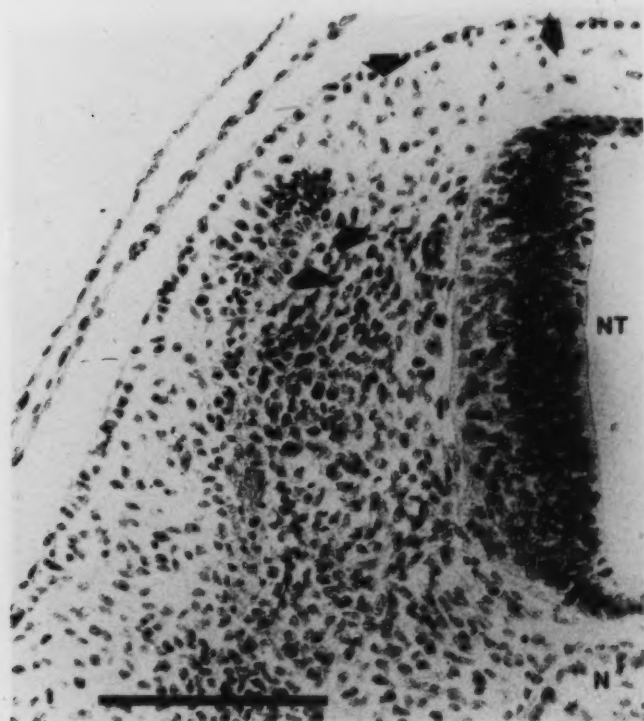
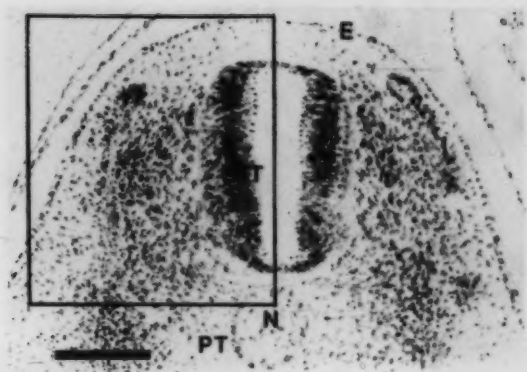
At this stage, a few quail mesenchymal cells were first observed in the space between the neural tube and notochord. This space was much narrower than the space between the notochord and aorta which had widened from earlier stages. A few widely separated cells were also first observed in the space dorsal to the neural tube. In previous stages, the neural tube had been in close association with the overlying ectoderm. In sections of embryo 38/5, a few isolated quail cells were observed within the chick mesenchyme of the developing limb bud. This embryo had received a transplant at somite 18. Spinal ganglia were located dorsally within the looser zone of the lateral sclerotome (rostral sclerotome half). These could usually be identified as distinct structures within the surrounding mesenchyme.

The condensed-cell regions in the lateral sclerotome each exhibited a distinct lateral projection which extended into the gap between two adjacent myotomes. In some instances a denser cell zone of the rostral portion of the next caudal lateral sclerotome was seen next to it. The intersomitic cleft or vessel still separated these two areas of similar density (Figure 16).

The space between the neural tube and the overlying ectoderm had increased by stage 18 and some quail cells could be seen occupying positions in this region (Figure 17). The cells remained very loosely associated and no pattern could be discerned. There were still only a few cells present in the narrow gap between the neural tube and the notochord.

Figure 17. Transverse section of a stage 18 embryo (33/1) showing quail cells dorsolateral to the neural tube

Section through the rostral portion of the quail somite. Note the quail cells (arrows) dorsolateral to the neural tube and within the adjacent chick somitic tissues, seen more clearly in the enlargement of the region indicated. Bar =100 μ m

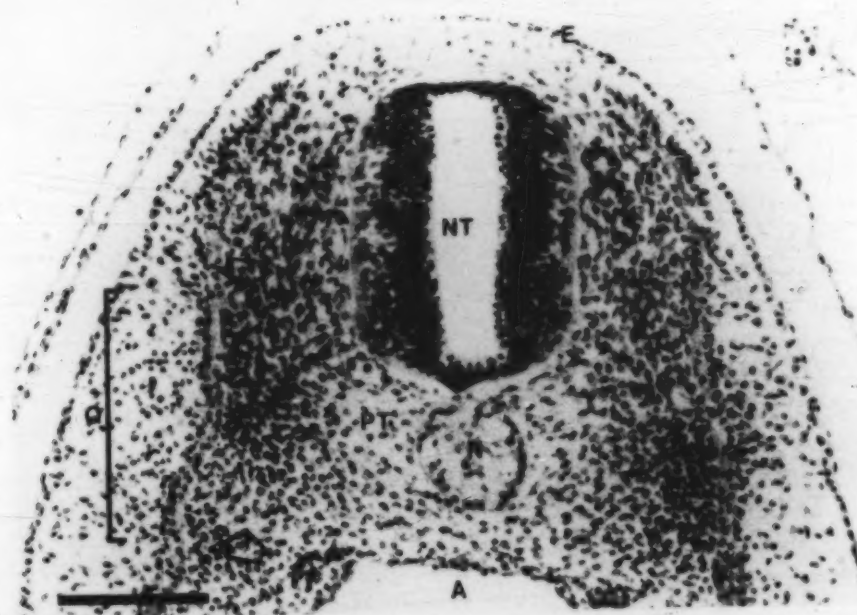
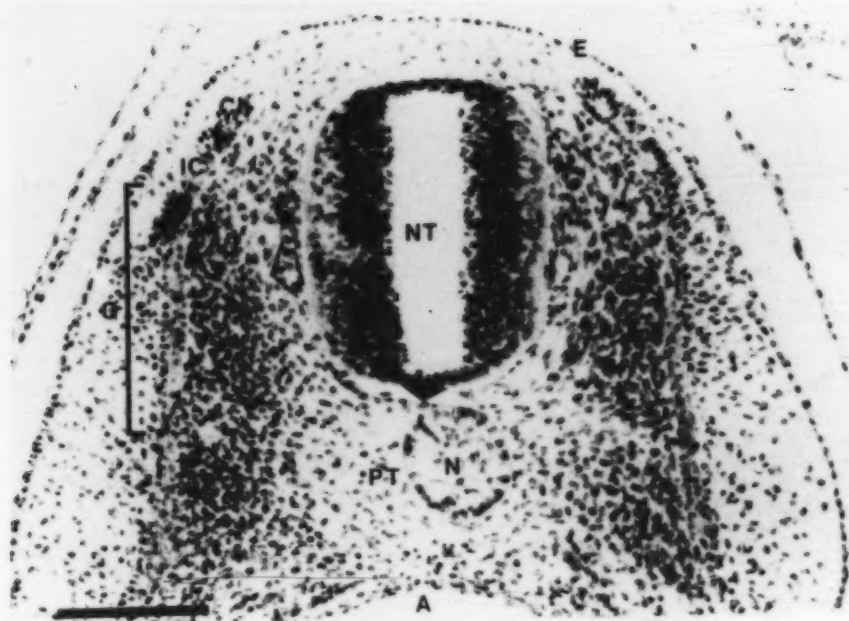


The intersomitic vessels continued to form the boundaries for most of the quail cells (Figure 18). Some isolated quail cells could be seen within the chick mesenchyme of adjacent ipsilateral somites (Figure 17). Some quail cells were also noted in the region of the chick myotome in the adjacent segment, but these cells could not be identified as myoblasts. Although adjacent dermamyotomes appeared to be joined, the junctions were still evident at the locations of the original intersomitic clefts (Figure 18). Generally there appeared to be less distinction between successive sclerotomes. Possible remnants of the intersomitic clefts within the sclerotome were observed in some regions, but at this stage they were not constant. Intrasclerotomic fissures also were not noted although differing cell-density patterns were quite apparent.

The various cellular condensations in the quail region became more distinct in successively older embryos (Figures 19, 20a, 20b). Dorsally in the lateral quail region, the caudal sclerotome half continued to be denser than the rostral sclerotome half which contained the spinal ganglion and nerve. In the perichordal tube, the denser zone came to occupy a mid-position (rostrocaudally) with loose-cell zones on either side which merged with adjacent loose-cell zones from the next sclerotomes (Figure 20b). Some intermingling of quail and chick cells could be seen in the perichordal tube, but this was minimal in embryos in which the quail zone filled one complete segment (Figures 20a, b). A greater number of displaced quail cells were observed within the lateral sclerotome regions. Of particular note was the continued presence of two densities in the lateral portion of the more

Figure 18. Transverse sections of a stage 18 embryo (33/1) showing vessels bordering the quail cell region

Middle (above) and caudal (below) parts of the quail region. Vessels (open arrows) border the quail region. A few quail cells (solid arrows) are present between the notochord and the neural tube. An intersomitic cleft (IC) is apparent above. Bar = 100 μ m



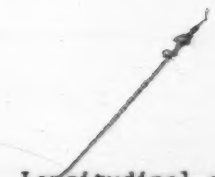


Figure 19. Longitudinal sections of a stage 23 embryo (53/5) showing cell condensation patterns

Cellular condensation patterns are demonstrated. Ganglia are separated by lateral densities adjacent to the neural tube (above). Ventrally (below) the perichordal tube densities are out of register with the lateral densities. Lower right shows the medial density centrally located within the quail region. Lateral densities contain quail cells (arrows) on adjacent faces with quail cell muscle filling the intervening gap. Bar = 100 μ m

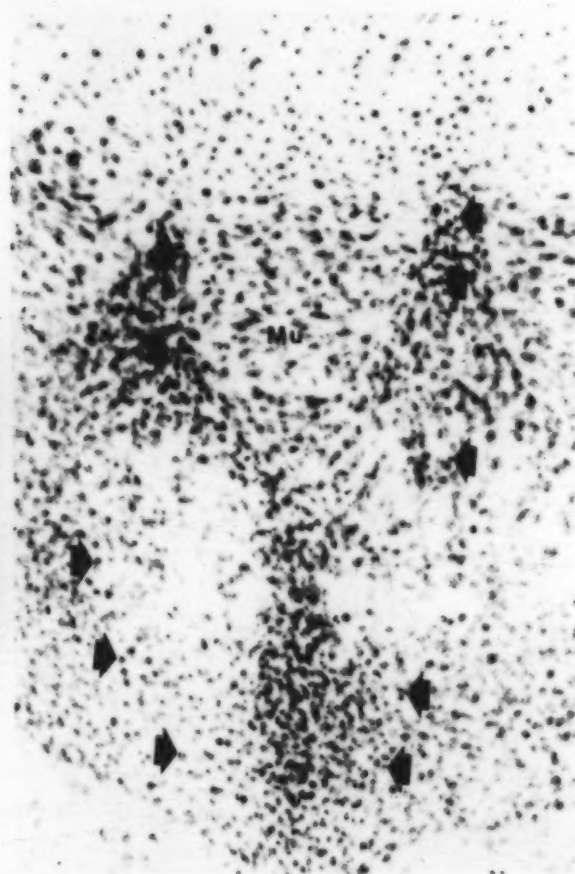
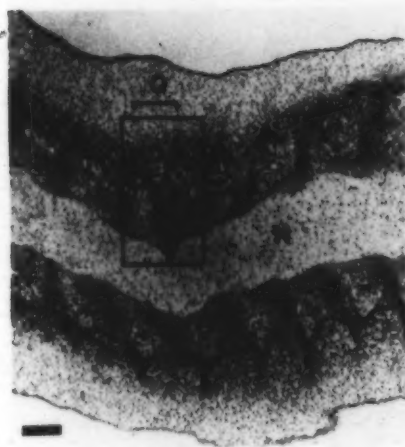


Figure 20a. Series of oblique sections through a stage 25 embryo (43/1) showing quail cell densities

Dorsal (above), middle (center), and ventral (below) sections through the quail region. The cell densities in the different areas can be noted. The quail area is indicated. Bar = 100 μ m

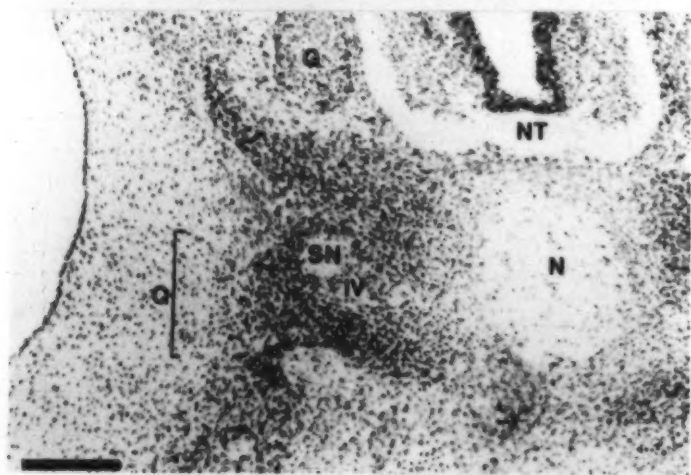
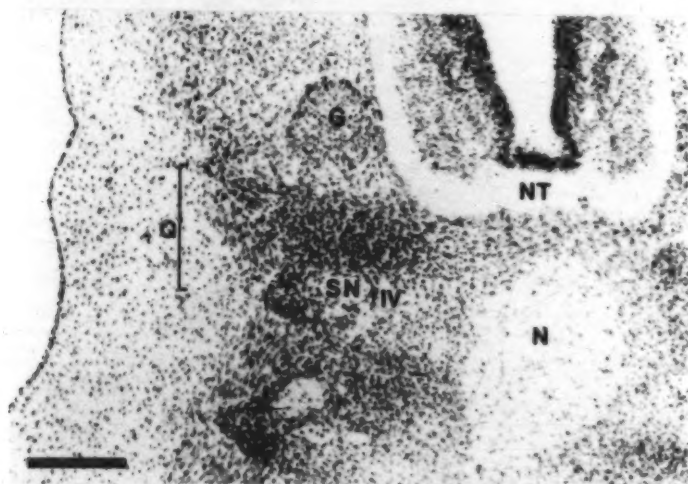
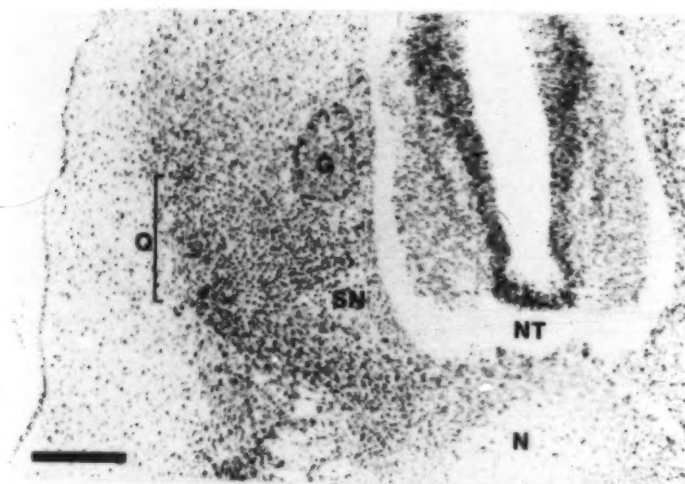
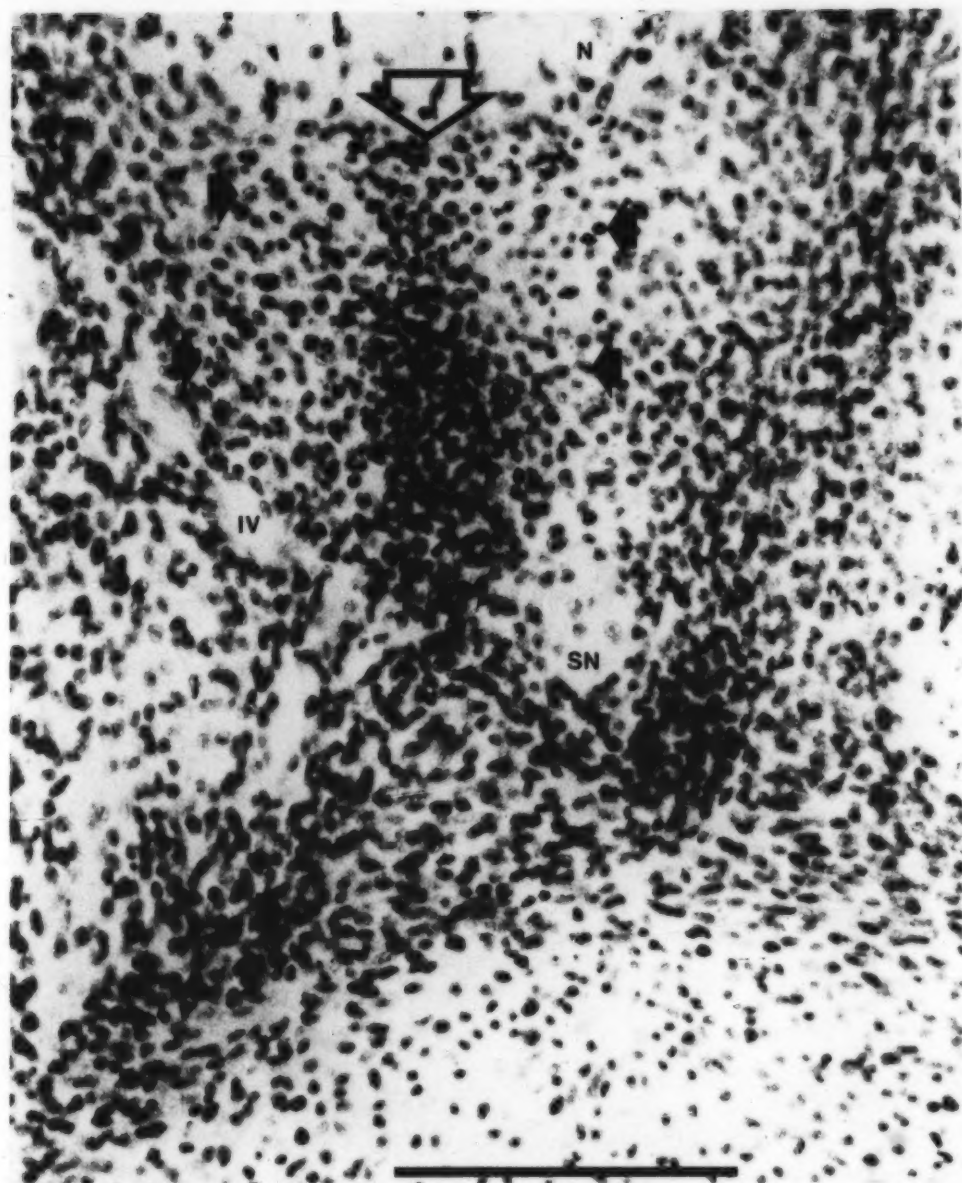


Figure 20b. Medial quail-cell densities in a stage 25 embryo (43/1)

This section is taken from a similar region as depicted in Figure 20a. Medial is to the right, lateral to the left. The axial quail region is illustrated. From a central density (open arrow) quail cells (solid arrows) can be seen both rostrally and caudally beside the notochord. Quail cells are also present in adjacent faces of the two lateral densities, and below in the dermis.

Bar = 100 μ m



ventral region. Quail cells continued to form adjacent faces of these two densities within the segment (Figures 19, 20a, 20b).

ii. Abnormalities

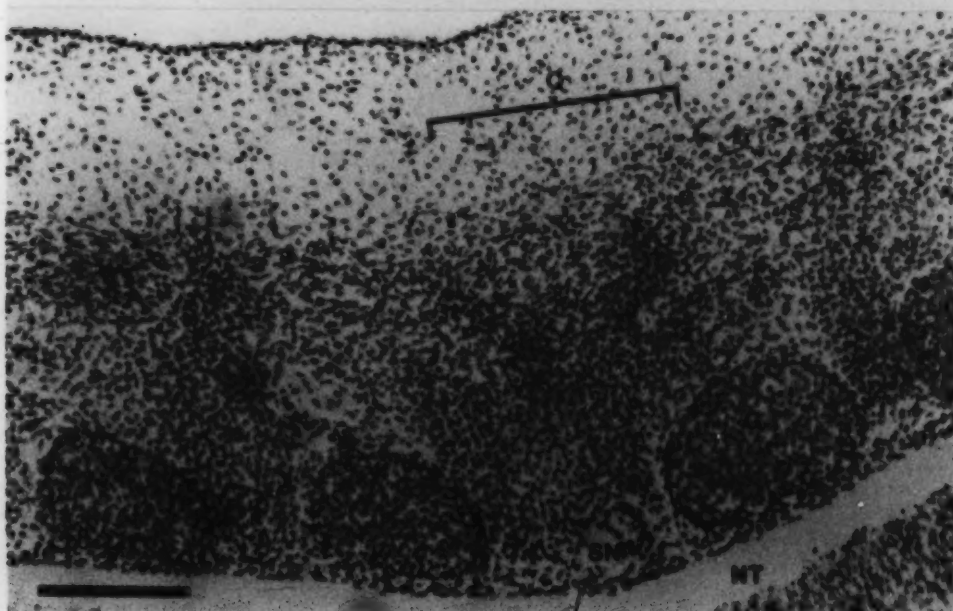
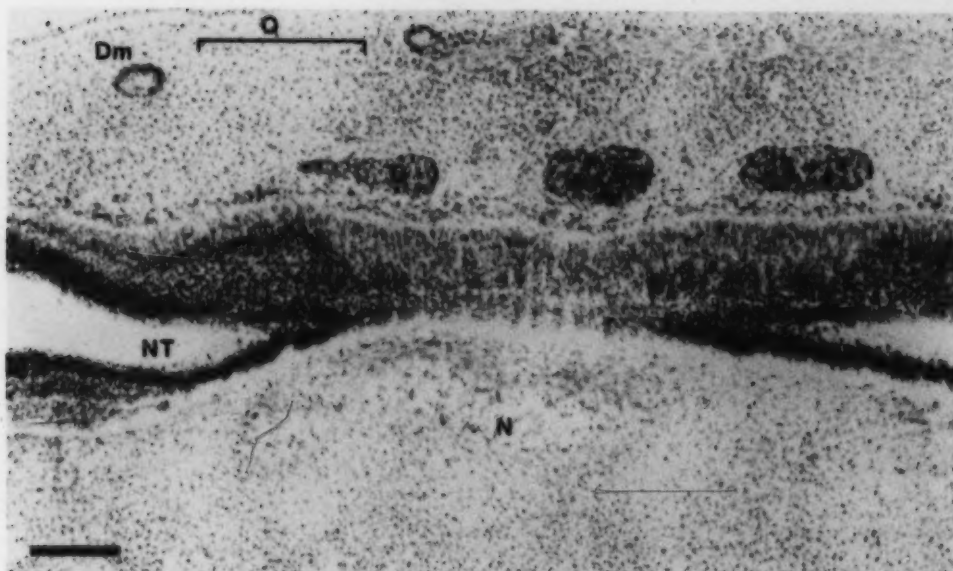
In 6 embryos a concavity was present toward the quail side and centered at the quail cell region. This abnormality was more apparent in embryos which had been sectioned longitudinally. In 5 embryos there were abnormalities noted in the spinal cord adjacent to the quail region. The notochord was constricted at the quail site in 2 embryos. In one embryo the notochord appeared to have been displaced to the non-quail side over several sections.

In addition to the 2 embryos in which cell death was evident, 5 embryos (29/3, 42/6, 54/4, 53/7, 54/2) demonstrated very little integration of quail cells into the axial tissues. Most of the area was filled by chick cells which generally exhibited a homogeneous distribution. Among the remaining embryos, cell-density patterns exhibited considerable variation. Abnormal locations of condensed-cell regions were associated with abnormal shape, size, or location of the spinal ganglion in the quail zone: the more severely disrupted the spinal ganglion morphology, the more irregular were the associated cellular condensation patterns. A wide variety of ganglion abnormalities were observed. Some examples of ganglion abnormalities have been illustrated in Figure 21.

In one embryo (29/4) there was a quail-cell ganglion present in the dorsolateral sclerotome in addition to a chick ganglion adjacent to the neural tube. Embryos in which there was an unusually large

Figure 21. Longitudinal sections illustrating ganglion and myotome abnormalities (55/3, 53/5)

Above, a deficiency of muscle/myotome can be seen. The ganglion is abnormal in shape and is fused to the next ganglion. Below, only a small spinal nerve is present within the quail region. Preceding sections showed a small ganglion in this same location. The adjacent ganglion is abnormally large and quail cells enclose the rostral half of it. Bar = 100 μ m



ganglion were deficient in segment numbers (i.e. one less segment was present on the quail side than in the contralateral side). There was also some overlap of quail cells into adjacent segments in association with abnormal ganglion size.

There was a small quail dermamyotome present in embryo 38/4. This had a very small chick dermamyotome interposed between it and the next caudal chick dermamyotome which was of normal morphology (Figure 16). The quail plus the small chick dermamyotome corresponded with a single chick dermamyotome on the contralateral side. The 5 embryos in which quail cell integration had been minimal either had no quail dermamyotome present or contained a very small amount of quail myotome which was inappropriately located and oriented. In some of these regions chick cells had partially filled in the deficient quail muscle. An example has been presented in Figure 21.

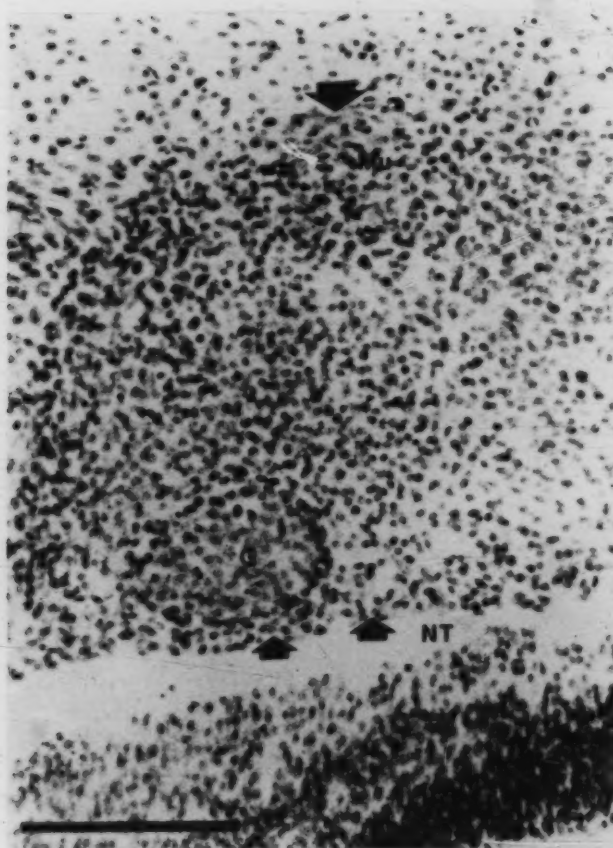
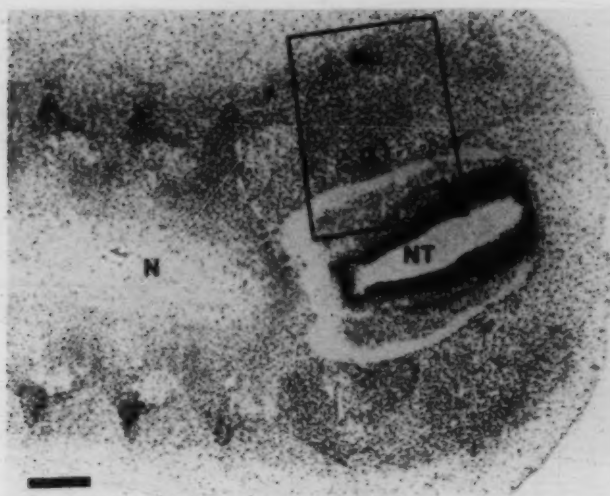
Among the 7 embryos in which a considerable amount of quail cell integration had occurred, there were still regions within the quail zone which contained groups of chick cells. The chick cells were most commonly observed in the perichordal tube and immediately adjacent to the spinal cord (Figure 22). Structures which appeared to be nephric ducts were observed within the sclerotome in 2 embryos.

iii. Summary of Intermediate Phase

During this phase of development, the pattern of cellular densities became progressively more pronounced, and several general observations could be made.

Figure 22. Longitudinal section illustrating presence of chick cells within the quail region (43/1)

Chick cells (arrows) can be seen abutting the neural tube within the quail region. Some chick cells are also possibly present within the quail muscle. Bar = 100 μ m



1. There was a small amount of mixing of chick and quail cells, in particular within the developing muscle and dermis.
2. Intersomitic vessels marked the boundaries of the quail somitic derivatives, but intersomitic clefts and intrasclerotomic fissures were not distinct.
3. The normal location of the spinal ganglion was in the rostral half of the quail sclerotome, lateral to the neural tube.
4. A condensed region of quail cells was present immediately caudal to the spinal ganglion.
5. Two laterally projecting densities were present at the rostral and caudal limits of the quail region, with quail cells forming adjacent faces and quail muscle between.
6. The perichordal tube condensations initially occupied a rostral position and later a mid-position within the quail region.
7. Abnormalities of the ganglion within the quail region occurred frequently and were associated with disruption of the cell-condensation patterns.
8. Abnormalities were associated with inappropriate somite matching in 5 cases.

C. Late Phase - Stages 26 to 36 (H & H)

There were 21 embryos in which some chondrification of cervical vertebral elements could be distinguished by morphological appearance. In these embryos, quail cells were present in the cartilage of the developing vertebrae, in the paraxial musculature, in the spinal cord meninges, in the dermis, and in the general connective tissue. The

somites replaced in this group ranged from somite number 8 (one embryo) to somite number 18 (one embryo). The difference in levels between the quail and chick somites varied from zero (8 embryos) to 7 (2 embryos) with 4 undetermined. The maturity difference between the two somites ranged from zero (7 embryos) to 3 (2 embryos) with 4 undetermined. This information has been summarized in Table 5.

Sections of 2 embryos (38/2, 95/4) exhibited extreme soft tissue necrosis. In both cases, however, the cartilaginous vertebral elements were distinguishable and the quail chondrocytes could be identified. Quail cell integration into the host embryo was minimal in 3 embryos (31/2, 38/6, 52/5) as most of the quail region was occupied by chick cells. Integration of quail cells in the other embryos was variable.

1. Somite Development

With few exceptions, the quail cells were found in regions only on the operated side of the embryo. What appeared to be single isolated quail cells were occasionally seen on the contralateral side of the embryo, although these may also have been necrotic chick cells. In a few cases, the quail cell region in the centrum extended slightly beyond the mid-sagittal plane. The notochord and spinal cord were misaligned vertically in the latter situations (Figure 23). In all embryos, the quail cell regions were continuous with the adjacent chick cell regions, demonstrating no interruptions of, or gaps between the two cell populations.

Along the craniocaudal axis, the various somite-derived tissues showed considerable variation in dispersion. The quail cells in the

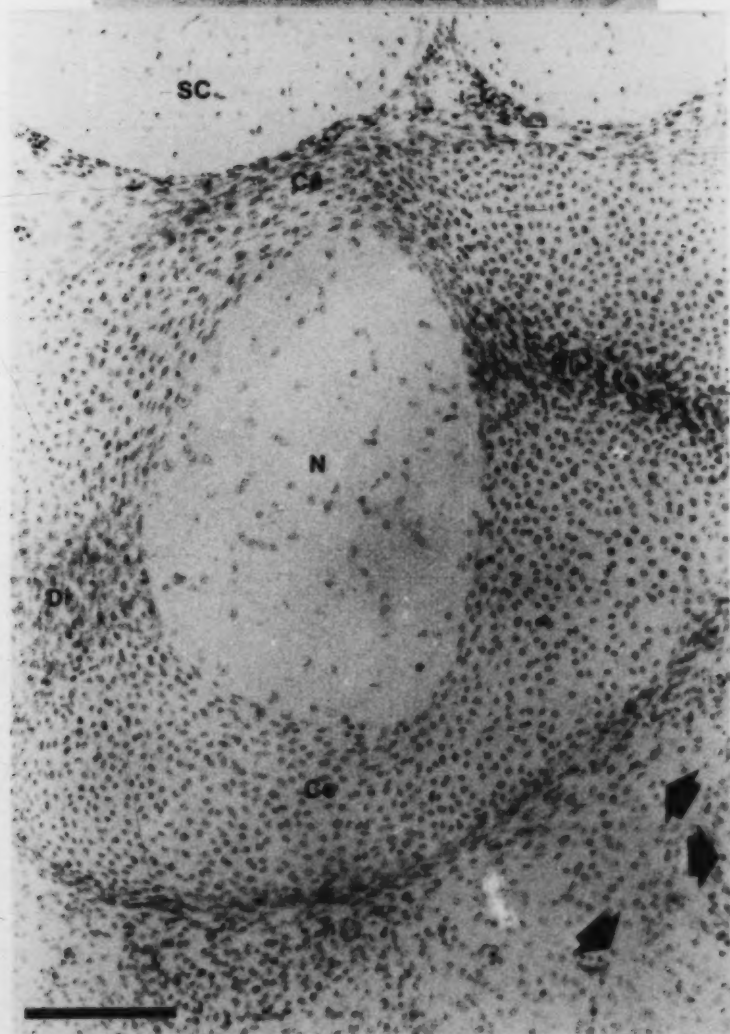
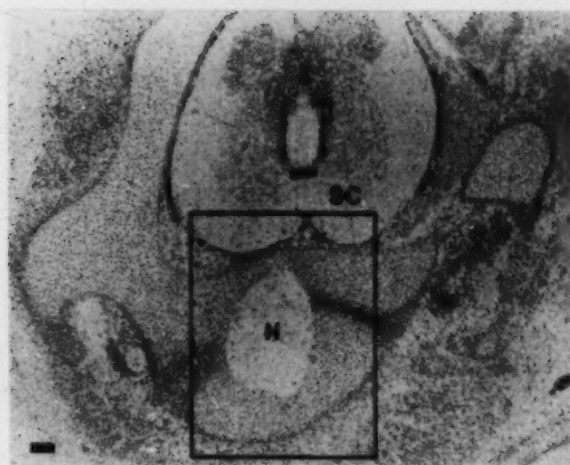
TABLE 5

SUMMARY OF SOMITE VARIABLES FOR
EMBRYOS OF STAGES 26 TO 36

Somite No. Removed		Embryo Numbers						
8	26/5							
9	34/4	40/4						
10	75/3							
11	40/1	92/3						
13	83/3	96/2						
15	31/3	38/6	52/5	83/1	96/5			
16	31/2	31/4	38/2	38/3	44/2	52/2	92/1	
18	95/4							
Difference in Somite Levels								
	0	1	2	3	7	u		
	0	92/1 95/4 96/2	38/2 38/2	31/2	31/4			
Difference	1	75/3 92/3	34/4		31/3			
in Somite	2	38/3 83/3		44/2		83/1		
Maturity	3	40/4				52/2		
	u						26/5 38/6 52/5 96/5	

Figure 23. Transverse section through the disc and adjacent centra of a stage 35 embryo (38/3)

Quail cells in the disc and two adjacent centra can be noted. Some mixing of chick and quail cells in muscle and connective tissue is apparent (arrows indicate quail cells). Medially, quail cells extend very slightly beyond the mid-sagittal plane of the spinal cord, but ventral to the notochord the chick cell region appears to have shifted correspondingly to the quail side. Bar - 100 μ m



forming vertebral elements were discretely localized, generally within two adjacent vertebrae and the intervening disc. Very little mixing of chick and quail cells was observed, except in cases where quail cell integration was minimal. This situation contrasted sharply with that of the composition of the paravertebral muscles. A large amount of quail and chick cell mixing was present in the vertebral musculature. Some quail cells were observed in the muscles up to two segments caudal to the quail vertebral levels. The quail cell population in the spinal cord meninges was located within the quail cell region in the vertebrae. The boundary of the quail meningeal area tended to be shifted further caudally in successive developmental stages. There was also some intermingling of chick with quail cells in the meninges. The quail cells located in the dermis were found in a generally localized zone although again there was some mixing of the two cell populations which increased towards the apparent dermatome boundaries.

Of the chimeras in this group, it was significant to note that the quail cells had formed portions of two consecutive vertebrae in all cases. In one embryo (31/2) the quail cell contribution to the more rostral vertebra was minimal. In the majority (16) of this group, the numerical difference between the position number of the somite which was replaced and the position number of the more caudal of the two involved vertebrae was "5" (Table 6). In 3 embryos the numerical difference was "4" and in 2 embryos the difference was "6".

TABLE 6

QUAIL REGIONS IN VERTEBRAE OF
EMBRYOS STAGES 26 TO 36

Embryo No.	Vert w Qu	#	Vertebral Components with Quail Cells									
			Cent	Disc	Ped	Lam	Spn	Cost	Tran	Artic	S	I
26/5	C2	5	*	*	u	u	u	*	u	u	u	
	C3		*	-	*	u	u	*	u	u	u	
31/2	C9	6	-	+	-	+	(-)	-	-	-	-	
	C10		*	-	*	*	(-)	*	*	-	*	
31/3	C9	5	*	*	*	*F	(*)	*	+	-	*F	
	C10		*	-	A	*F	(*)	*	*	*F	*	
31/4	C9	6	*	*	*	*F	(+)	*	*	-	*F	
	C10		*	-	A	*F	(*)	*	*	*F	+	
34/4	C3	5	*	*	+F	+	(-)	+	*	-	*F	
	C4		*	-	*F	*	(-)	*	*	*F	*	
38/2	C10	5	*	*	*	*	(-)	+	+	-	*	
	C11		*	-	*	+	(-)	*	*	*	-	
38/3	C11	6	*	*	+	*F	*F	+	+	*	*	
	C12		*	-	*	*F	*F	*	*	*	-	
38/6	C9	5	*F	*	-	*	+	+	+	-	*F	
	C10		*F	-	*	*	+	+	*	*F	-	
40/1	C5	5	*F	*	+F	*	(+)	+	-	-	*F	
	C6		*F	-	*F	*	(*)	*	*	*F	-	
40/4	C4	4	+F	*	*F	*F	(-)	A	*	-	*F	
	C5		*F	-	*F	*F	(*)	*	*	*F	*	
44/2	C10	5	*F	*	-	*	-	-	-	-	*F	
	C11		*F	-	*	*	-	*	*	*F	-	
52/2	C10	5	*	*	-	*F	-	*	-F	-	*F	
	C11		*	-	*	*F	-	*	*F	*F	-	
52/5	C9	5	*F	*	+	+F	-	+	-	-	+F	
	C10		*F	-	+	+F	-	*	A	+F	-	

TABLE 6 (continued)

Vertebral Components with Quail Cells											
Embryo No.	Vert w Qu	#	Cent	Disc	Ped	Lam	Spn	Cost	Tran	Artic	
											S I
75/3	C5	4	*	*	-	*	*F	+	-	-	*
	C6		*	-	*	*	*F	*	*	*	-
83/1	C9	5	*	*	+	+	(*)	+	-	-	*
	C10		*	-	*	*	(*)	*	*	*	-
83/3	C7	5	*	*	+	+	(*)	+	+	-	*
	C8		*	-	*	*	(*)	*	*	*	-
92/1	C10	5	*	*	*	+	-	*	*	-	*
	C11		*	-	*	+	-	A	A	*	-
92/3	C5	5	*	*	*F	+F	(-)	+	*F	-	*F
	C6		*	-	*F	*F	(-)	*	*F	*F	+
95/4	C12	5	*	*	*	*F	-	*	*	-	-
	C13		*	-	A	*F	-	A	*	-	-
96/2	C7	5	*F	*	*	*F	*F	*	*	-	*F
	C8		*F	-	A	*F	*F	A	A	*F	-
96/5	C9	5	*	*	-	*F	*F	+	-	-	*F
	C10		*	-	*	*F	*F	*	*	*F	-

Vert w Qu = vertebrae containing quail cells

= somite number minus number of the caudal vertebra

Cent = centrum

Ped = pedicle

Lam = lamina

Spn = spinous process

Cost = costal process

Tran = transverse process

Artic = articular process

S = superior

I = inferior

C = cervical

* = many quail cells forming significant portion of the element

+ = some quail cells present and confluent with other quail areas

- = no quail cells or very few isolated quail cells present

() = element not yet formed

A = element absent

F = abnormal fusion of elements

u = undetermined

11. Quail Cell Composition of Vertebrae

Although some variation in quail cell content was apparent among the 21 embryos included in this group, a number of common features were noted indicating a distinct distribution of the quail cells. Examination of the vertebral processes individually permitted identification of the more commonly occurring dispersal patterns for each of these elements. Quail cell content in each of the vertebral elements for all embryos in this group has been summarized in Table 6.

a. **Centra and Disc.** Quail cell dispersal patterns were most consistent in the centra. Twenty of the 21 embryos contained quail cells which comprised some or most of the adjacent quadrants of two consecutive centra, and most or all of the corresponding half of the intervening disc (Figures 23, 24, 25, 26). In some embryos a variable amount of chick cells occupied medial regions within the quail zone. In the more caudal vertebra quail cells first appeared in the ventral perichondrium at about mid-centrum on the operated side of the embryo. Progressing rostrally, the quail region extended dorsally, eventually to enclose the half of the notochord on the operated side, and then reached into the neural arch. In the single embryo (31/2) containing quail cells in only one centrum (C10), those cells were located in the rostral right quadrant of the involved vertebra and in the rostral disc (of C9). In centra which demonstrated good integration of quail cells, and which were morphologically the most "normal" in appearance, the quail cell area could be delineated illustrating the continuity across the two vertebral levels.

Figure 24. Transverse section through the disc and adjacent centra of a stage 36 embryo (75/3)

Quail cells are forming the right half of two adjacent centra and the intervening disc. Some quail cells in the perichondrium extend to the left of the spinal cord. A few quail cells are possibly forming the meninges (arrows). Bar = 100 μ m

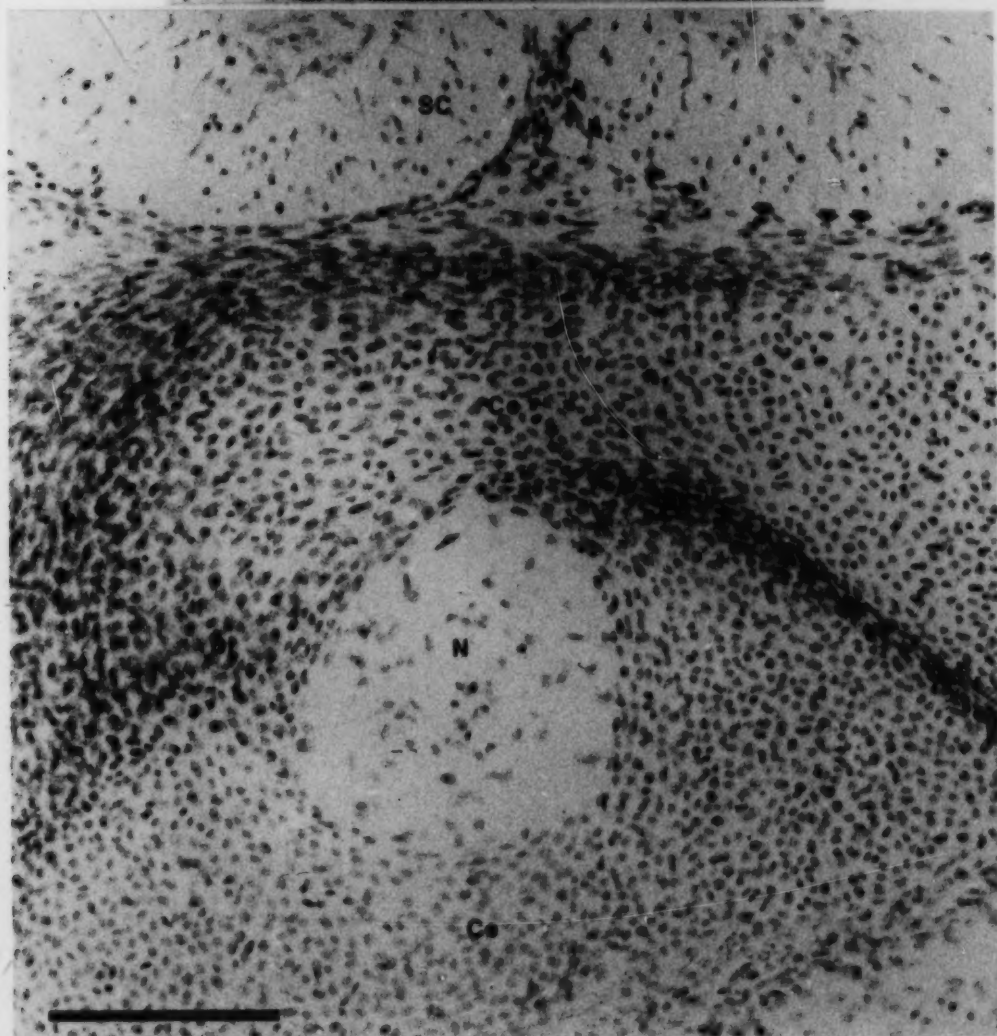
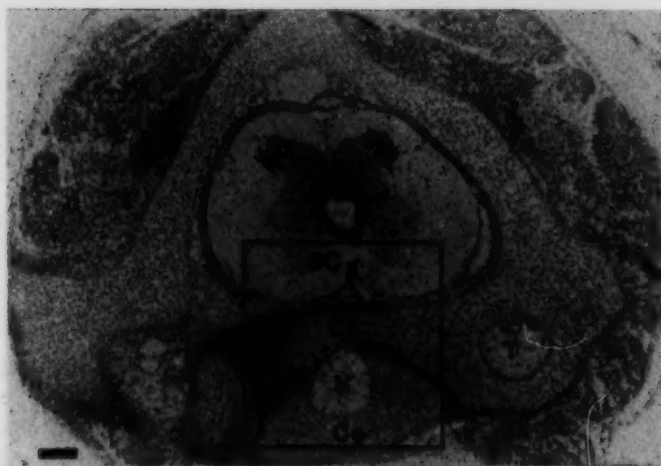


Figure 25. Transverse sections through the centra of stages 35 (26/5) and 30 (52/2) embryos

Quail cells are forming quadrants of two adjacent centra and the intervening half-disc in each of these embryos. Above, quail cells are also present in the pedicle of the caudal vertebra. The rostral vertebra is at bottom in each case. Bar = 100 μ m

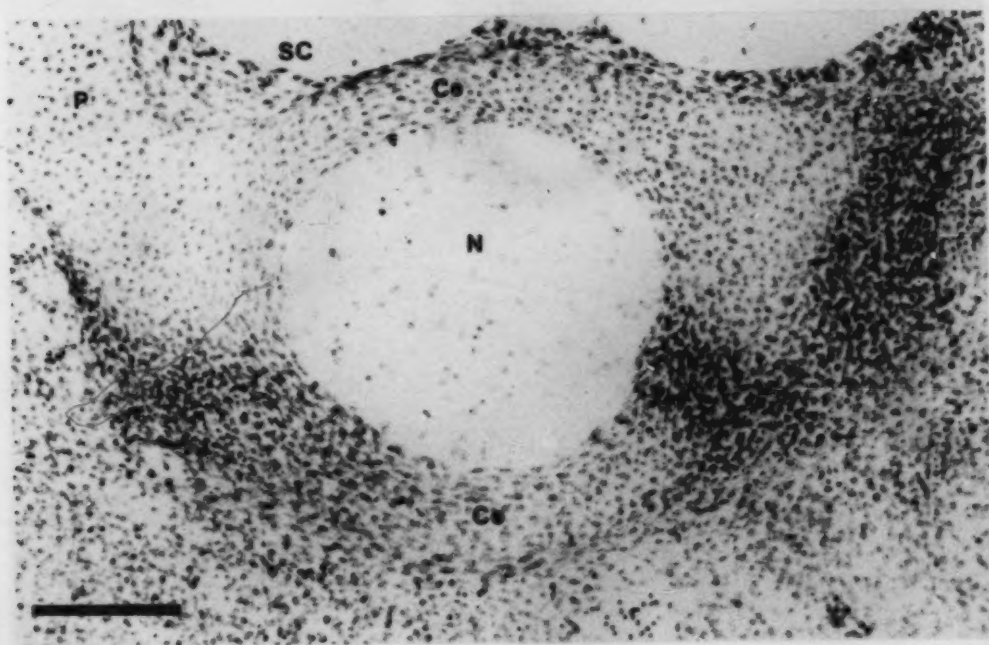
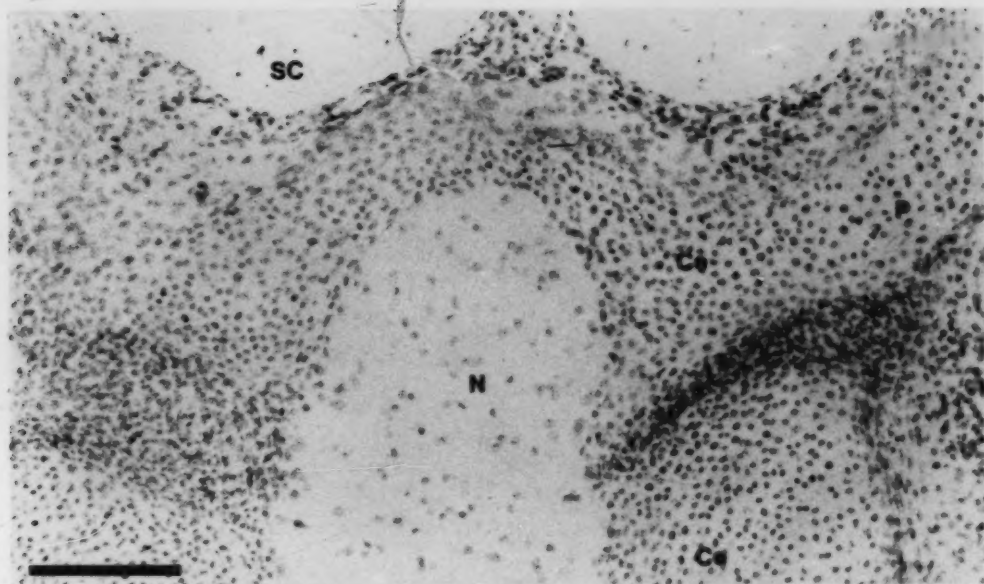
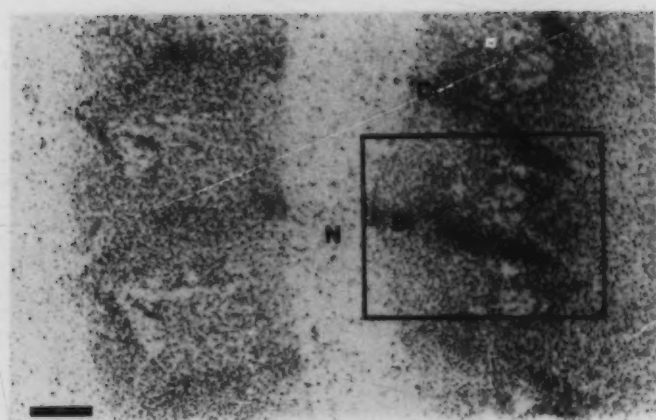


Figure 26. Longitudinal section through the centra of a stage 27 embryo (83/3)

Quail cells are in the right half of the disc and the adjacent centra. Lateral condensations are the transverse processes. The process in the quail area is that of the caudal vertebra, and is almost entirely quail-celled. The rostral process contains no quail cells, but a few are close to it. Bar = 100 μ m



b. Pedicles. Ten of 20 embryos contained quail cells in the pedicles of the same (as for centra) two adjacent vertebrae. Of these 10, only 2 exhibited relatively normal morphology of the two pedicles and continuous distribution of the quail cells. In 2 embryos the quail cell content was high but the pedicles were fused. Five embryos displayed a large proportion of quail cells in the caudal pedicle but only a few quail cells in the rostral pedicle. Two of these 5 had fused pedicles. One embryo contained a few quail cells in each pedicle. Of the remaining 10 embryos, 6 contained a large proportion of quail cells in the caudal pedicle and none in the rostral pedicle; 4 contained quail cells in the rostral pedicle and the caudal pedicle was missing. Eighteen of the 20 embryos either contained few or no quail cells in the rostral pedicle, or exhibited a major abnormality of the pedicles (both fused or caudal pedicle absent). Whenever the caudal pedicle was present, it contained quail cells (16 out of 16 embryos). Some examples of quail cell content in the pedicles have been presented in Figures 27 and 28.

c. Laminae. Quail cells were present in portions of both of the adjacent laminae in 20 out of 20 embryos. Two of these have been depicted in Figure 29. Twelve embryos contained a large proportion of quail cells in both laminae, 8 of these demonstrating at least partial fusion of these elements. The caudal lamina contained numerous quail cells and the rostral lamina only a few quail cell in 5 embryos, one of these exhibiting laminar fusion. The reverse situation (many quail cells in the rostral lamina and a few quail cells in the caudal

Figure 27. Longitudinal section through the pedicles of a stage 27 embryo (83/3)

Pedicles and transverse processes occupy the spaces between successive ganglia. The caudal pedicle and transverse process are almost entirely quail-celled. The rostral pedicle (above) has a few quail cells along the adjacent border (arrows).
Bar - 100 μ m

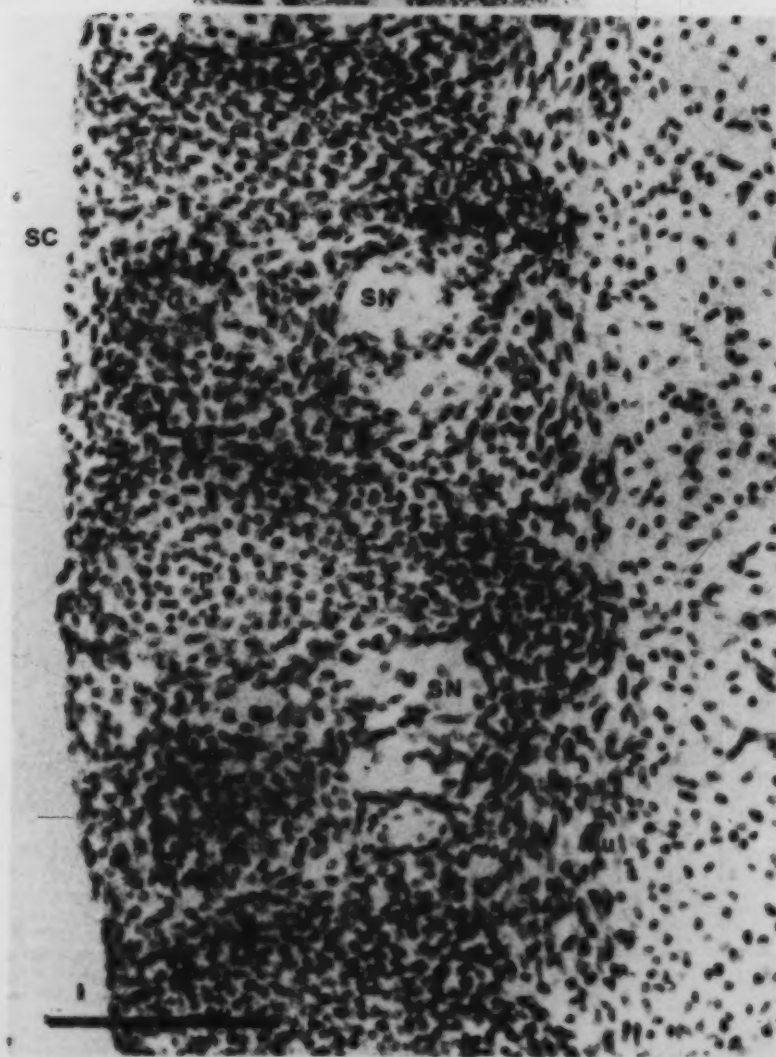
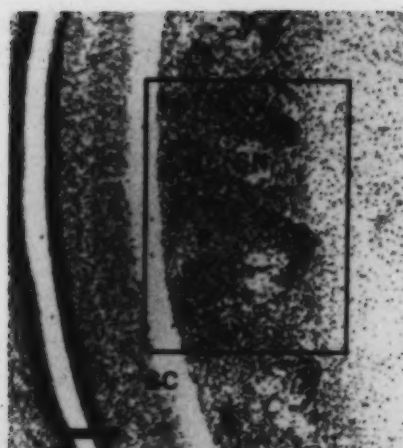


Figure 28. Longitudinal section through the pedicles of a stage 28 embryo (40/1)

Note that the caudal pedicle (right) is entirely quail, while the rostral pedicle (left) has a few quail cells (arrows). A small ganglion is present between the pedicles. Bar = 100 μ m

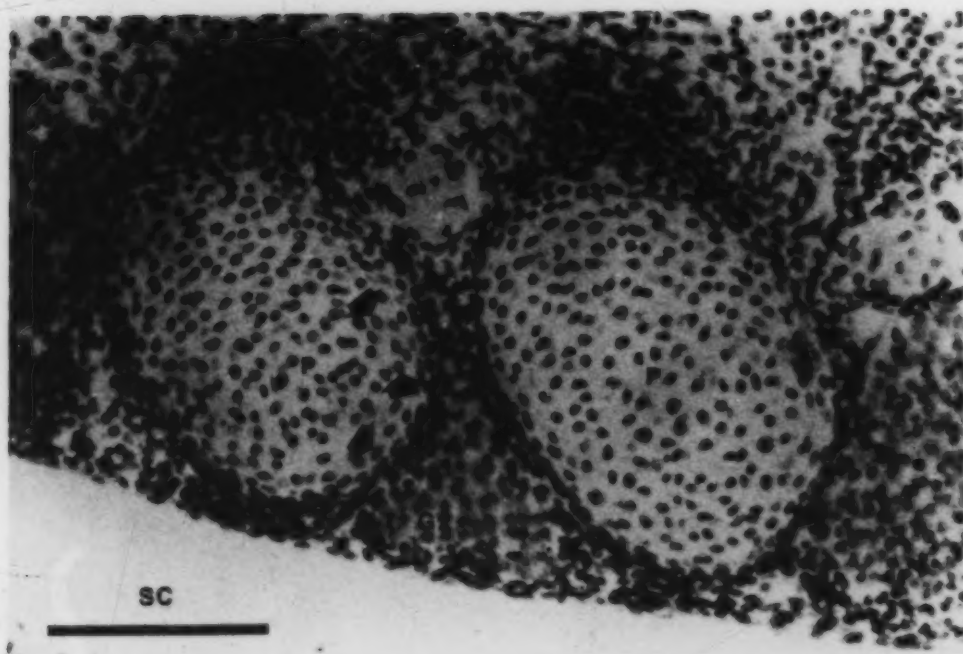
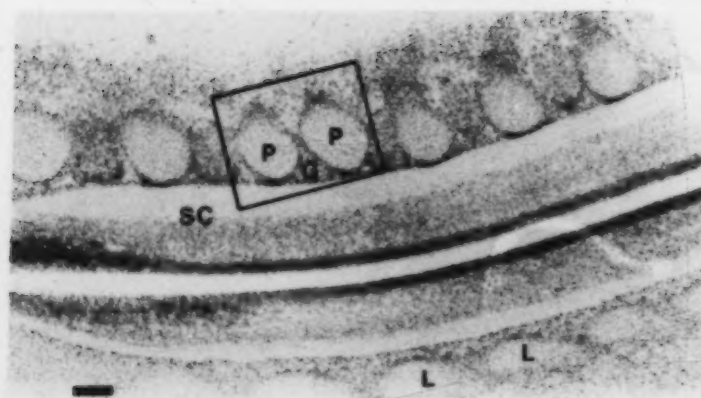
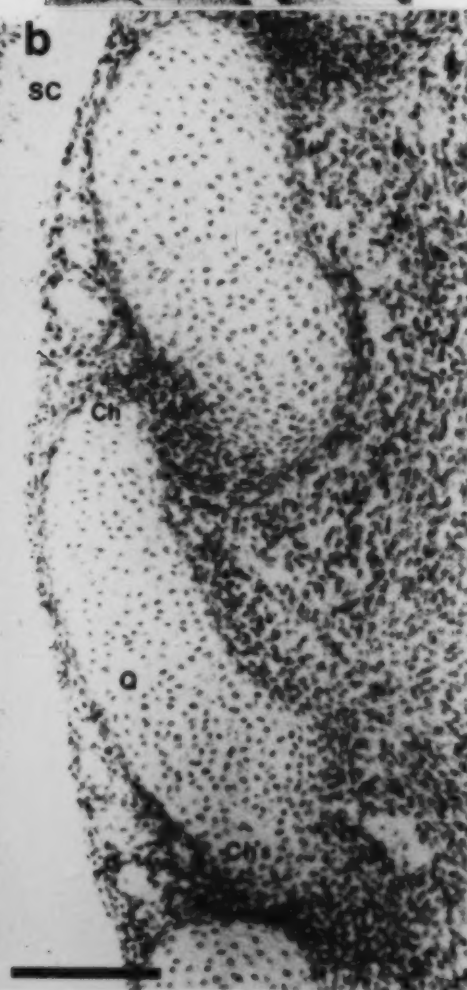
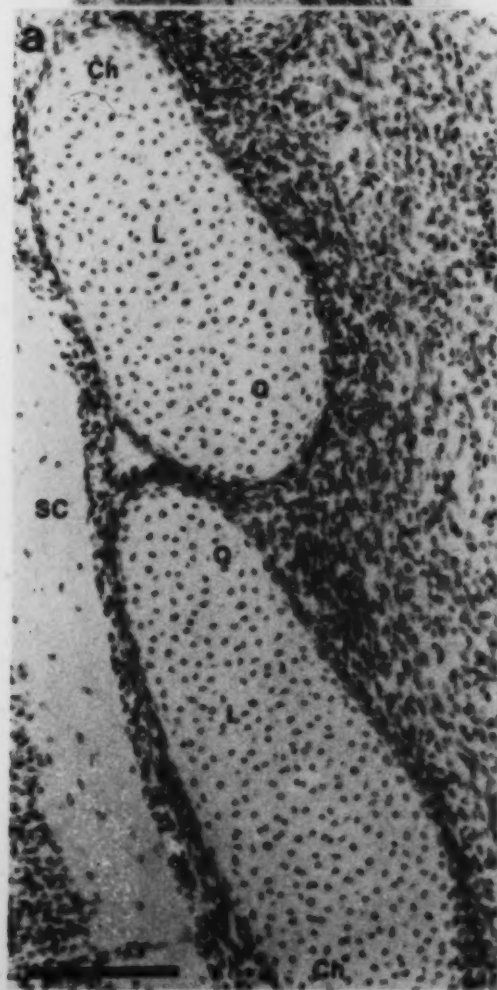
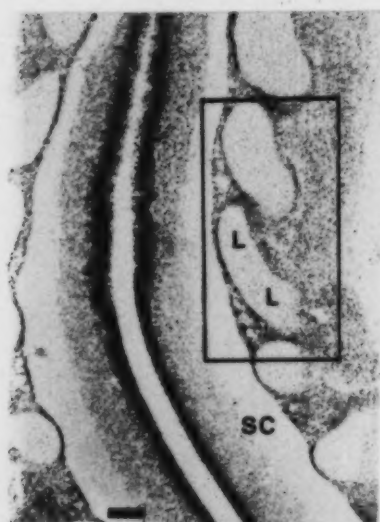
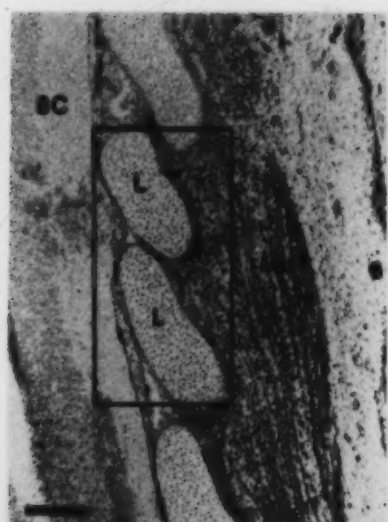


Figure 29a. Longitudinal section through the laminae of a stage 35 embryo (44/2)

Quail cells occupy portions of two adjacent laminae, both smaller than any of the chick laminae. Chick cells are present in the extremities of the two quail-containing laminae. Bar = 100 μ m

Figure 29b. Longitudinal section through the laminae of a stage 32 embryo (96/2)

The two involved laminae are fused, the majority of cells being quail. Chick cells are present at each extremity. Note the wider dispersal of quail cells (arrows) in the adjacent mainly chick-celled muscle. Bar = 100 μ m



lamina) was present in only one embryo. Two embryos contained only a small amount of quail cells in each of the laminae.

d. Spinous Processes. The dispersal patterns in the spinous processes were inconsistent. This occurrence was possibly related to the high incidence of abnormalities associated with these vertebral elements, and to the lack of chondrification in 10 embryos. In the latter cases, quail cell involvement in formation of these processes could only be approximated based upon presence of quail cells in the region where the spines should have been forming. In 9 embryos quail cells were present in neither of the two spinous processes or their precursors. Seven embryos contained a large proportion of quail cells in both spines or precursors. Of these 7, 4 embryos displayed fusion of the two spinous processes. The remaining 3 embryos had not yet formed spinous processes on the involved vertebrae. In 3 embryos, quail cells were present to a large extent in the caudal spine region, but only minimally or not at all in the rostral spine region. One embryo contained some quail cells in each of the two spinous processes. Some of the spinous processes containing quail cells have been shown in Figure 30.

e. Costal Processes. Fifteen of 21 embryos demonstrated quail cells in each of the costal processes or rudiments of the two adjacent vertebrae. Of these 15, 11 cases were found in which the rostral costal process contained quail cells only in the inferiorly projecting tip (Figure 31). In the remaining 6 embryos, 4 were missing one of

Figure 30. Transverse sections through the spinous processes of stages 35 (38/3) and 36 (75/3) embryos

Quail cells can be seen on the right side in the lamina and extending to the dorsal midline in the spinous processes. Above, the neural arch has fused dorsally. Below, a small gap persists at this level. Bar = 100 μ m

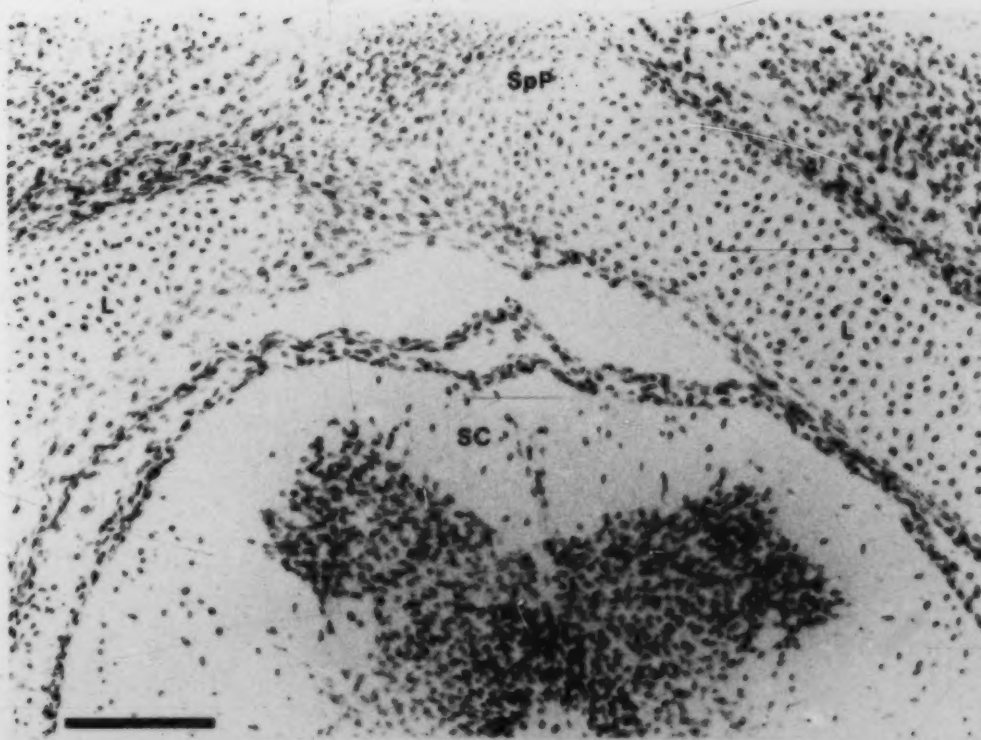
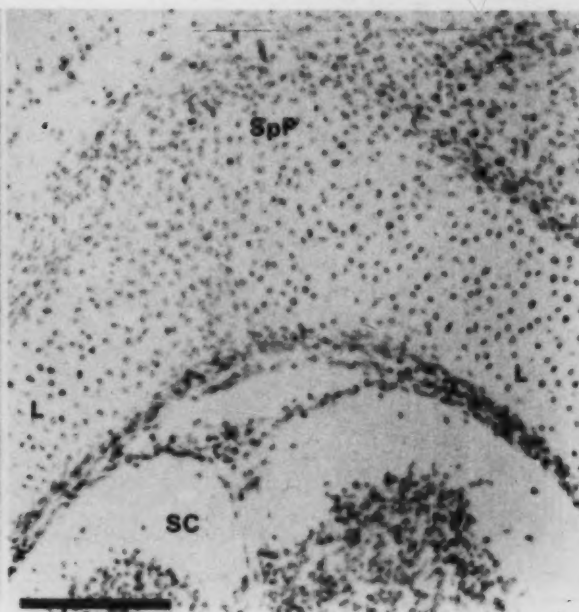
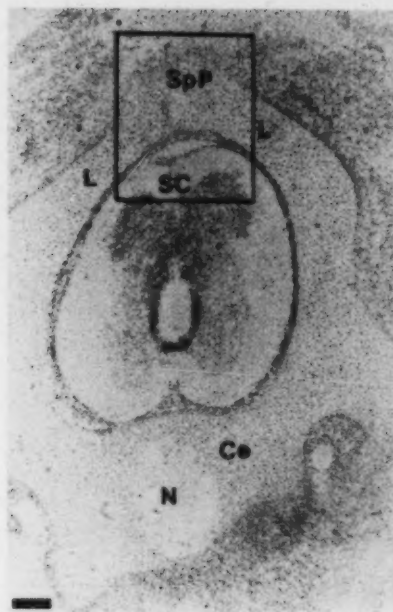
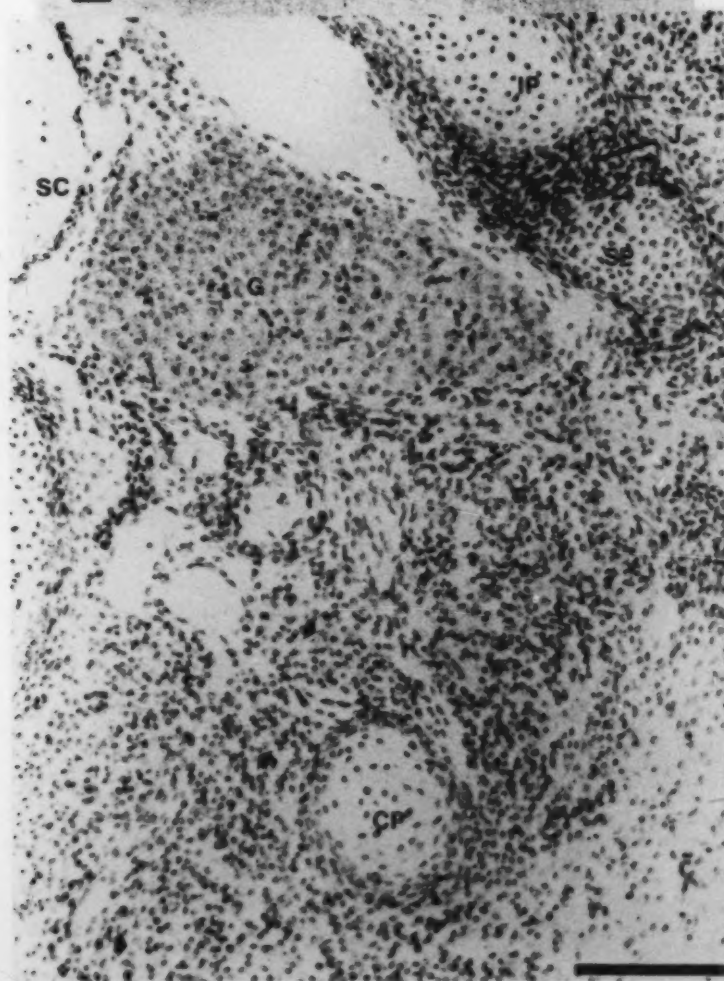
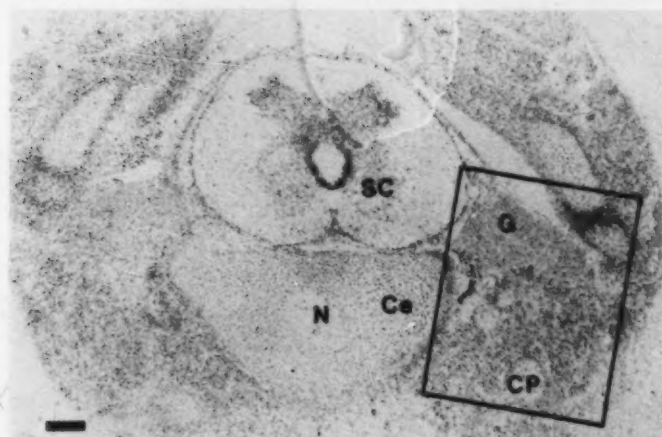


Figure 31. Transverse section through the costal and articular processes of a stage 36 embryo (75/3)

The tip of the costal process contains quail cells only dorsally. The facet joint is formed of quail cells, as are the two articular processes. The majority of muscle is composed of quail cells, but some chick cells (arrows) may be present. Bar = 100 μ m



the two costal processes (the existing costal process containing a large number of quail cells), and 2 exhibited quail cells only in the caudal costal process (Figure 32).

f. Transverse Processes. Adjacent transverse processes contained at least some quail cells in 10 of 20 cases. In one of these embryos the transverse processes were fused. Seven of the remaining 10 embryos demonstrated quail cells in only the caudal transverse process, although the rostral process was present. Each of the other 3 embryos was missing the transverse process of the caudal vertebra. In 15 of 20 embryos examined, either few (5 cases) or no (7 cases) quail cells were found in the rostral transverse process, or the caudal transverse process was absent (3 cases). As was the situation for the pedicles, whenever the caudal transverse process was present, it was found to contain quail cells (17 of 17 embryos) (Figures 33, 34).

g. Articular Processes. Eighteen of 20 embryos contained quail cells in the adjacent articular processes (i.e. the inferior articular process of the rostral vertebra and the superior articular process of the caudal vertebra) (Figures 31, 35). In 12 of these cases, the involved elements demonstrated at least partial fusion. Quail cells were noted in the inferior articular process of the caudal vertebra in 6 embryos. Quail cells were not present in the superior process of the rostral vertebra in any of the embryos. Of the 2 embryos without quail cells in adjacent articular processes, embryo 31/2 was the single case exhibiting a divergent pattern of quail cell distribution in the

Figure 32. Longitudinal section through the centra and costal processes of a stage 35 embryo (44/2)

Two costal processes are depicted. The caudal costal process (right) is entirely quail, while the rostral costal process (left) is entirely chick. Quail muscle crosses the gap between the two costal processes. The associated centra are fused.
Bar - 100 μ m

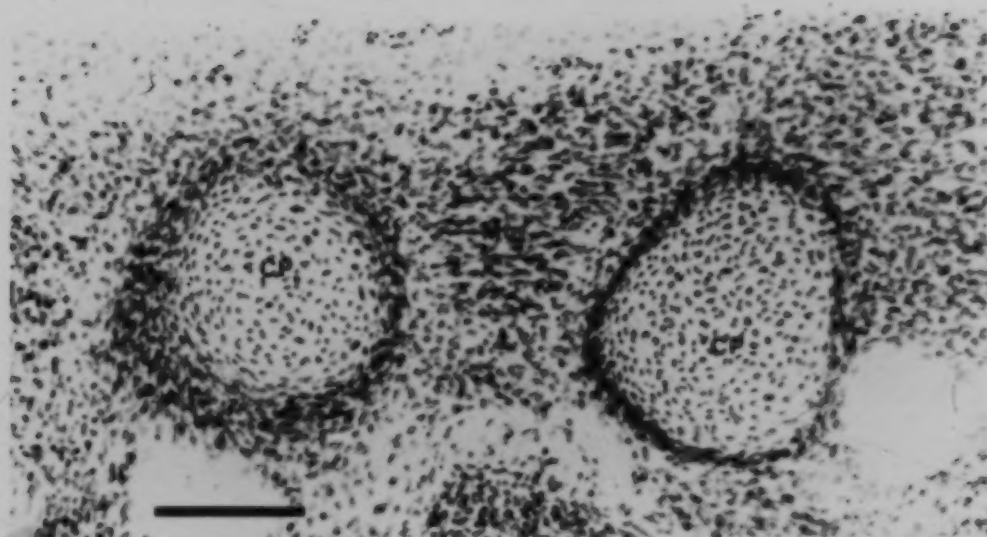
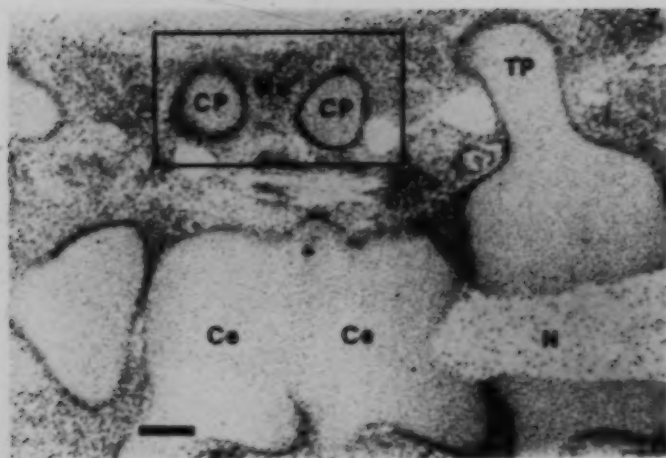


Figure 33. Transverse section through the transverse and costal processes of a stage 36 embryo (75/3)

The transverse foramen is entirely enclosed by quail cartilage. Quail cells are also present in the region of the developing meninges (open arrows), and in the connective tissue. Some quail cells may be forming the vertebral vessels (solid arrows).
Bar - 100 μ m

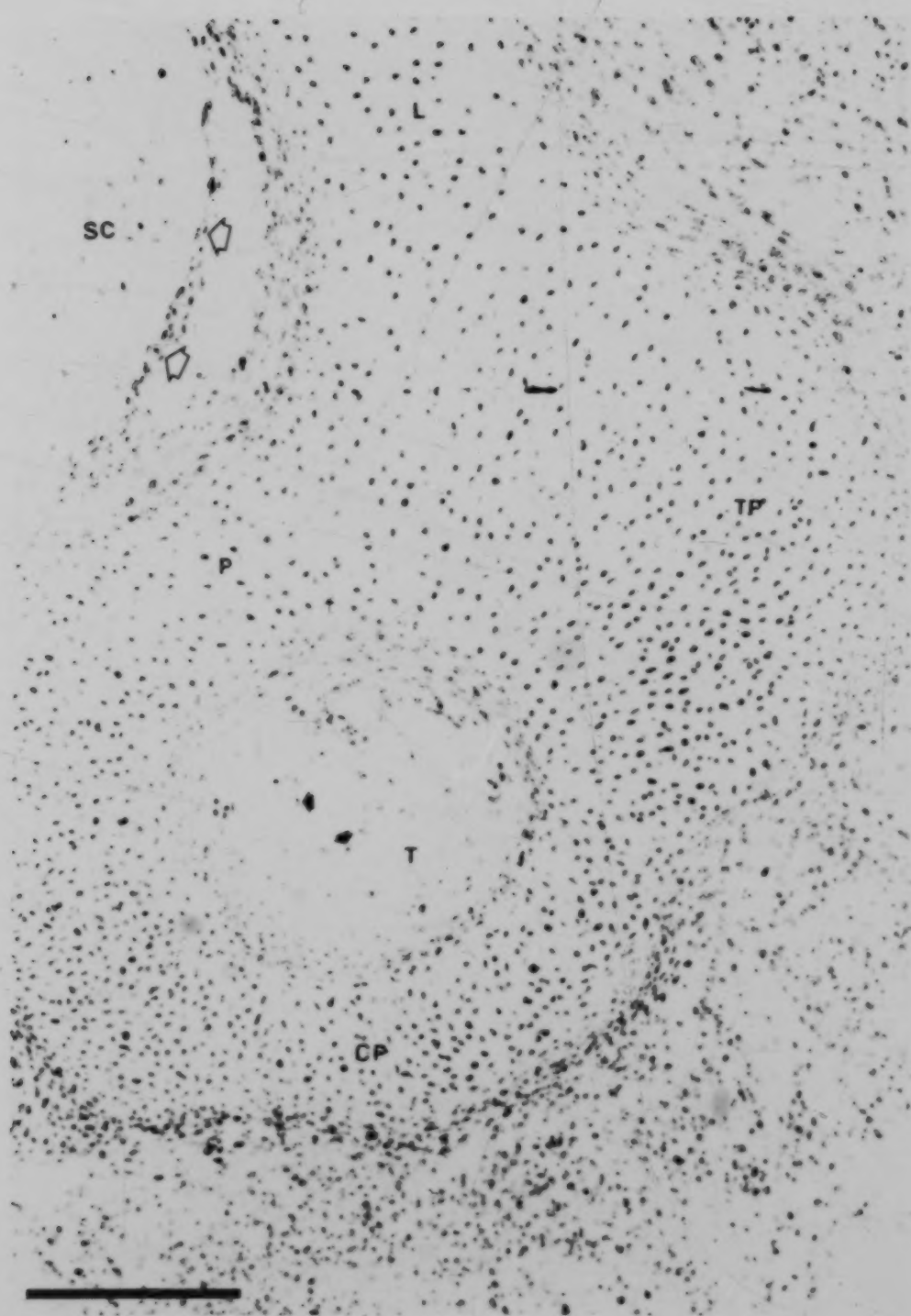


Figure 34. Oblique section through the transverse process and lamina of a stage 35 embryo (26/5)

Portions of the pedicle, lamina, and transverse process are presented. The pedicle and transverse process are entirely quail. The lamina is quail ventrally and chick dorsally at this level. Arrows mark the chick-quail junction in the perichondrium. Bar - 100 μ m

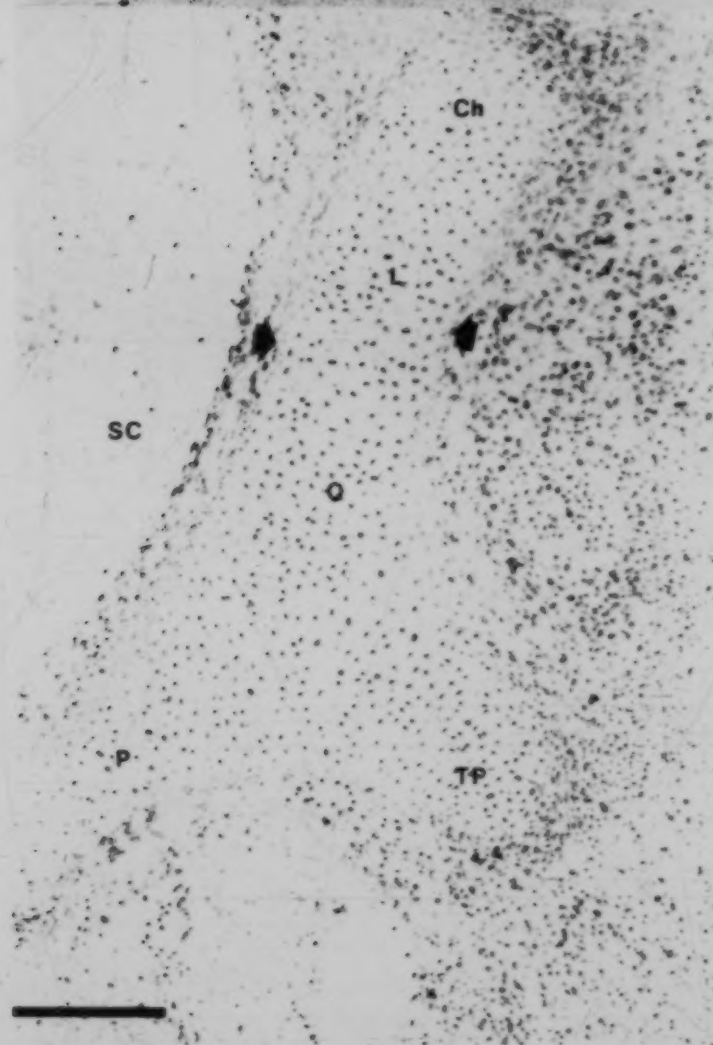
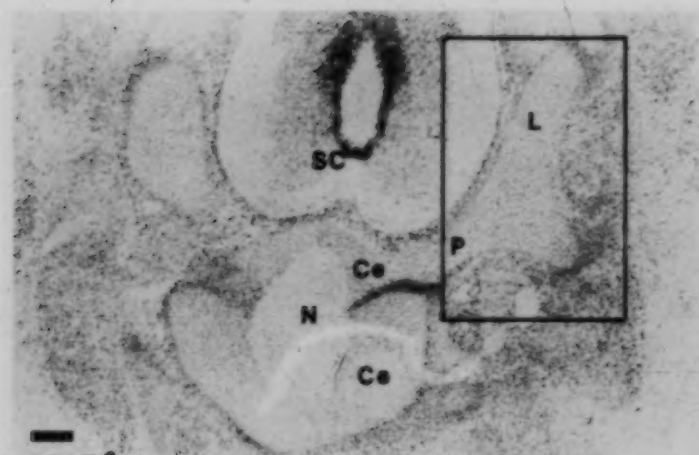
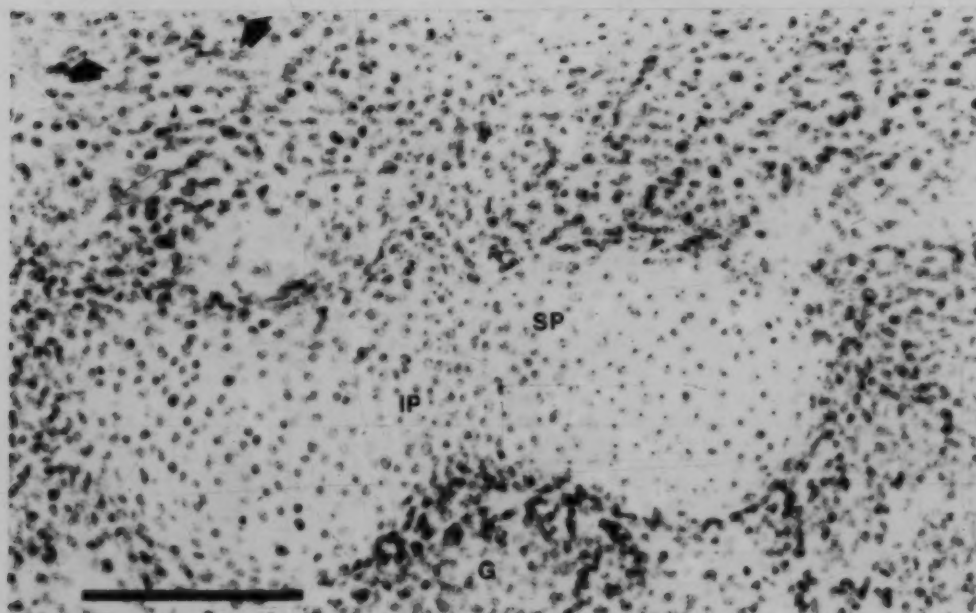
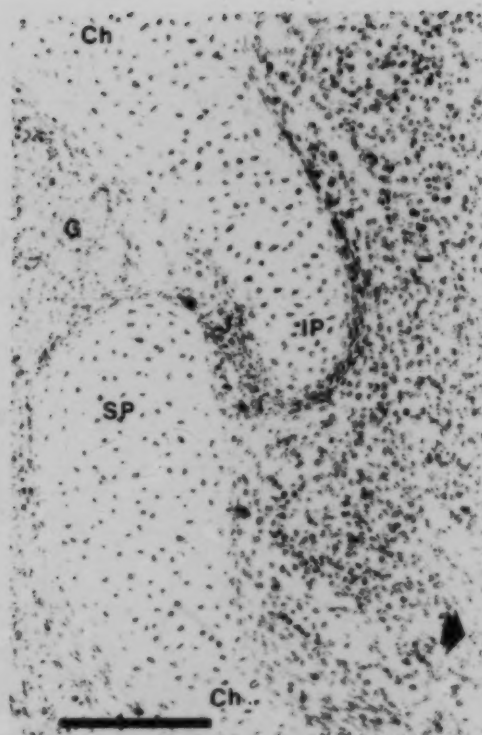
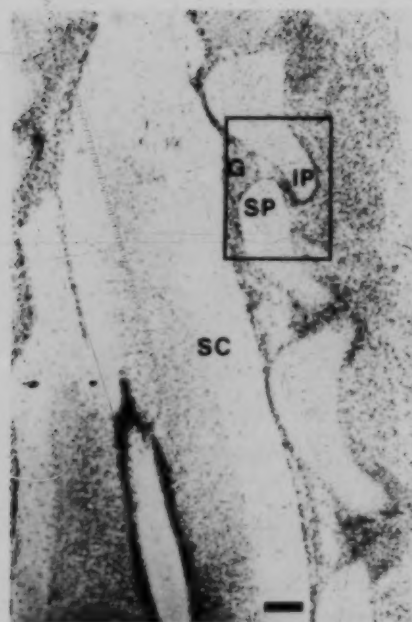


Figure 35. Longitudinal sections through the laminae and articular processes of stages 35 (44/2) and 28 (40/1) embryos

Above, adjacent articular processes and the joint space are quail. Chick cells are present in the neural arch extensions. Below, fused articular processes show quail continuing rostrally (left). Note chick-quail mixing in the muscle (arrows indicate quail cells). Bar = 100 μ m



centra, and embryo 95/4 contained no quail cells in any of the articular processes.

iii. Abnormalities

A wide variety of abnormalities was observed in the vertebral elements and in adjacent soft tissues within the quail cell area. Additionally, some disruption of the vertebrae in adjacent regions or on the contralateral side of the embryo had occurred in a few cases. No single embryo exhibited completely normal morphology of all of the structures and tissues in the operated region.

Some of the abnormalities observed in the vertebrae have been mentioned previously. Abnormalities generally included intervertebral fusion, appearance of anomalous cartilaginous processes, reduced proportions of elements, and complete absence of some parts of the involved vertebrae. The specific elements which were fused or absent for each embryo have been noted in Table 6 (p 126). Examples of abnormalities have been presented in Figure 36.

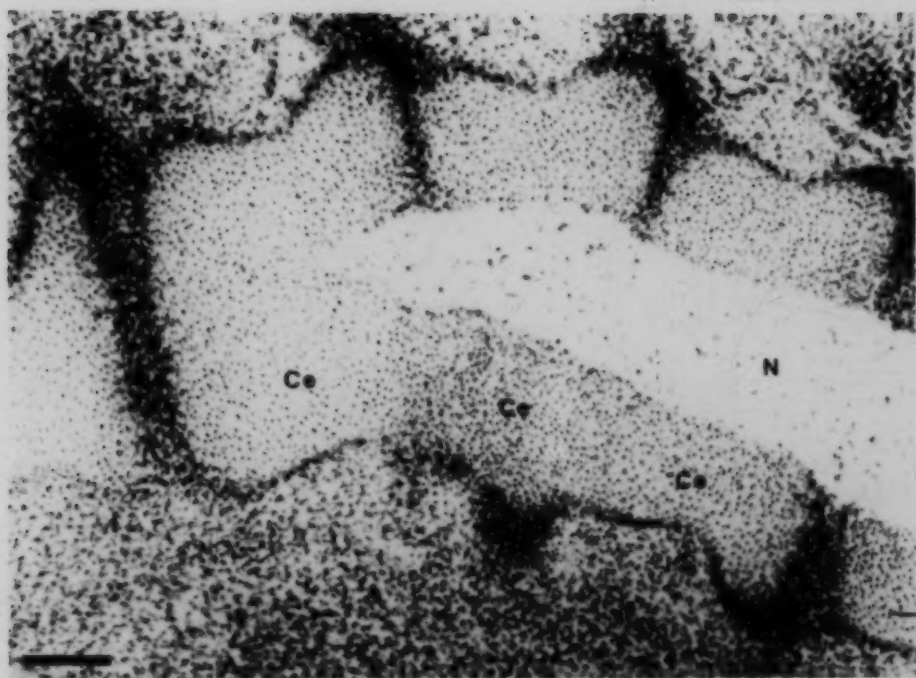
There were numerous soft tissue anomalies. The most frequently occurring of these were abnormalities of the spinal ganglia within, and often in the next segment to the quail site. Inappropriate size or position of spinal ganglia was observed in 17 embryos. The types of abnormalities observed included absence or mis-positioning of the ganglion; ganglion smaller or larger than normal; ganglion fused with the adjacent caudal ganglion; and ganglion broken up into several smaller ganglia. These anomalies frequently occurred in conjunction

Figure 36. Longitudinal sections of stages 30 (52/5) and 32 (29/6) embryos illustrating centra abnormalities

Above, the next rostral centrum is fused with the two quail-containing centra on the quail side. The right side of the middle vertebra is shorter than the left side creating a lateral flexure. Many chick cells are present in the quail region.

Below, the quail side of the caudal vertebra is deficient creating a wedge-shaped centrum. The rostral vertebra has a transverse process attached to the caudal border on the quail side. On the contralateral side the transverse process is attached to the rostral border.

Bar = 100 μ m



with each other and were always associated with an abnormality of some part of the neural arch.

Distinct spinal cord abnormalities were present in 2 embryos (40/4, 44/2). In one embryo the spinal cord at the quail site seemed to be composed of numerous vesicular structures. In the second embryo, some extraneous tissue was present within the central canal of the spinal cord. Each of these correlated with the occurrence of damage to the neural tube during the transplant procedures. The notochord was constricted at the quail-cell site in 4 embryos. In each case a cellular condensation was present creating the appearance of a wedge projecting from the disc region into the normal plane of the notochord. The notochord seemed to be displaced laterally to the non-quail side in 5 embryos.

The paravertebral muscle was notably deficient in 6 embryos. In some instances there was a distinct gap in the muscle band at the quail site. In other cases the quail muscle region was of diminished proportions as compared to adjacent chick muscle regions. Frequently some amount of chick-cell muscle had partially filled in the deficiency. In 4 embryos structures resembling nephric ducts were present in the paravertebral region. The cells forming these structures were quail in 3 cases and chick in one case.

A variable amount of chick tissue was observed within the quail-cell region in 14 embryos. Usually small groups of chick cells were located immediately adjacent to the spinal cord and/or the notochord. In some embryos the chick cells accounted for the greater proportion of cells within the quail region. In the remaining 7 embryos, chick cell

infiltration into the vertebrae within the quail region was negligible, with possibly a few isolated chick chondrocytes scattered in the cartilage.

iv. Serial Reconstructions

For 9 of the embryos in this group, serial reconstructions of the vertebrae were made. The embryos were selected based on several criteria:

1. Quail cell content - a large proportion of chick cells in the quail region was assumed to indicate poor quail somite integration.
2. Morphology of the vertebrae - embryos in which many elements were absent or fused were considered to be abnormal. Some examples of abnormalities, however, were present in the specimens selected.
3. Plane of sectioning - to depict different views of the quail content in the vertebrae, some transversely and some longitudinally sectioned embryos were chosen.
4. Stage of the embryo - embryos were selected from different stages in this group such that a progression of development could be observed.

The embryos which were selected for serial reconstruction were: 38/3, 38/6, 40/1, 44/2, 52/2, 75/3, 83/1, 83/3, and 96/5. Three which were considered to be representative of the group have been presented in Figures 37 to 42.

Figure 37. Serial reconstructions of C6 of embryo 75/3 (stage 36)

Caudal views (a, c) and rostral view (d) of the caudal vertebra. Quail region has been shaded separately (b) and within the vertebra. Note bilateral differences in the various vertebral elements (discussed in the text). Bar = 100 μ m

In e and f, successive sections have been separated 3mm to demonstrate more clearly the location of the quail cells. The quail region begins part way through the centrum and continues dorsally and laterally to encompass most of the pedicle and costal process, all of the transverse and superior articular process and part of the lamina and spinous process.

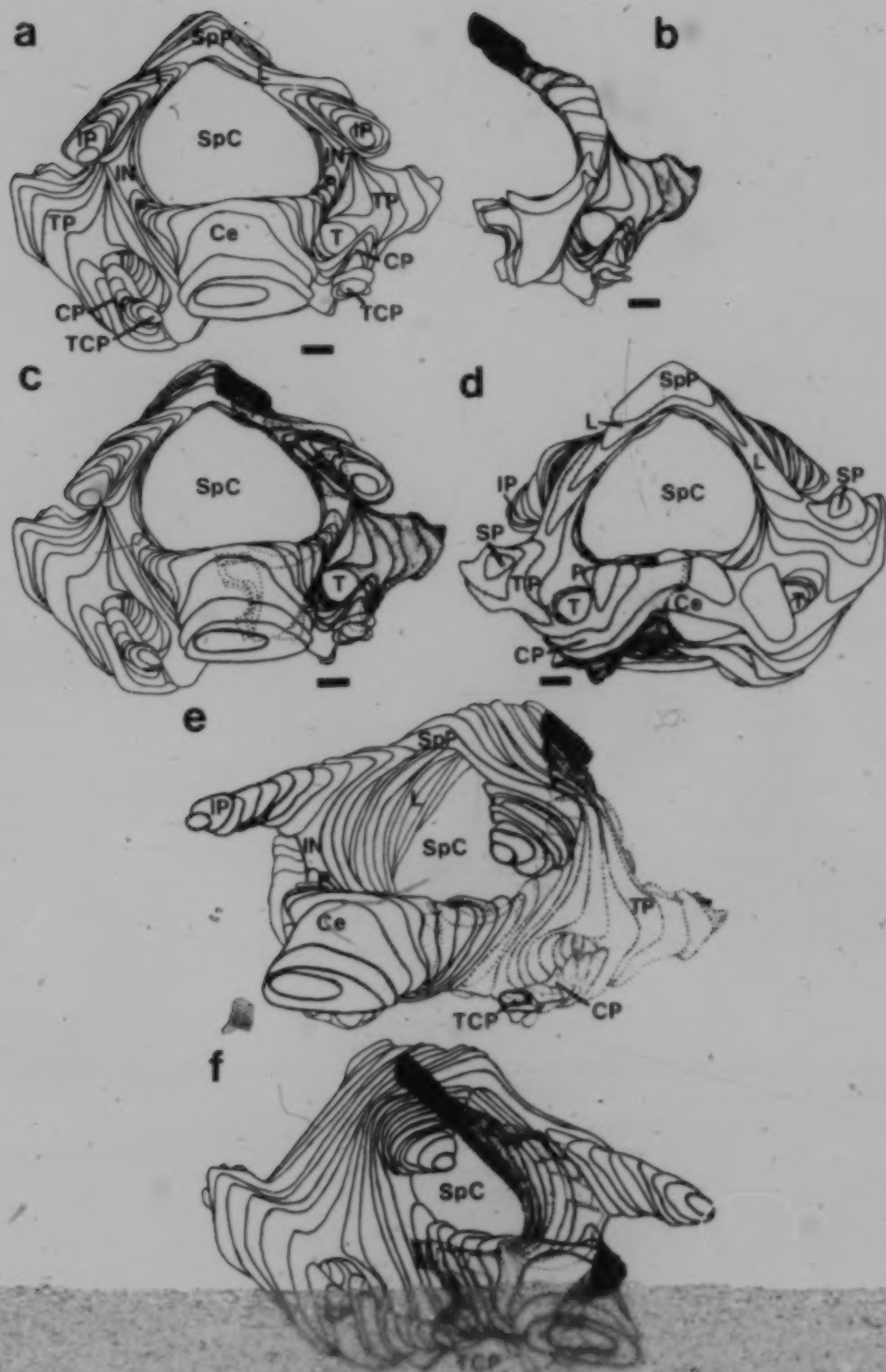


Figure 38. Serial reconstructions of C5 of embryo 75/3 (stage 36)

Caudal (a, c) and rostral (d) views of the rostral vertebra. Note that the quail region (b) includes part of the tip of the costal process, but the centrum and neural arch quail regions are discontinuous. Bilateral differences in shape are again apparent. Bar = 100 μ m

In e and f, successive sections have been separated 5mm to demonstrate more clearly the location of the quail cells. The quail region recedes dorsally through progressively more rostral sections. The location of quail cells in the dorsal portion of the tip of the costal process can be noted.

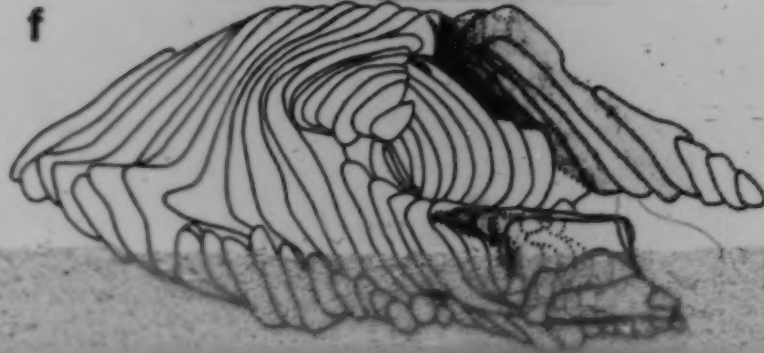
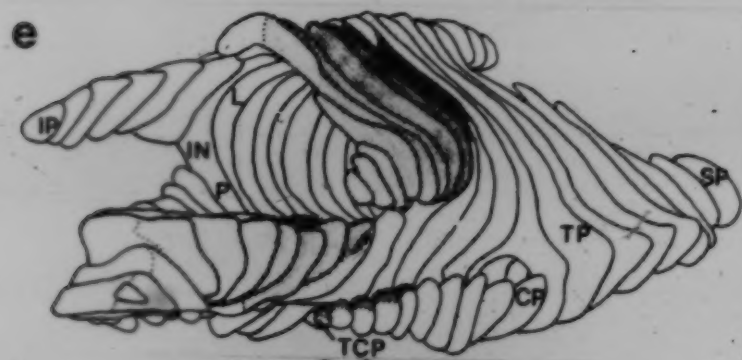
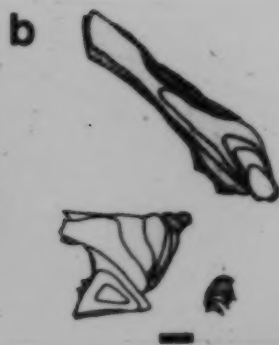




Figure 39. Separated right caudal view of C5 and C6 of embryo
75/3

Successive sections of C5 and C6 have been separated 10mm to illustrate the continuity of the quail cell region. C6 is outlined in black to distinguish it from the C5 elements. The region of partial fusion in the lamina is indicated by a broken line (arrow).



Figure 40. Serial reconstructions of C11 of embryo 52/2 (stage 30)

Caudal (above left), rostral (above right), and caudal separated views of the caudal vertebra are presented. The pedicle and transverse process of the quail side were joined to the centrum of C10. Bar = 100 μ m

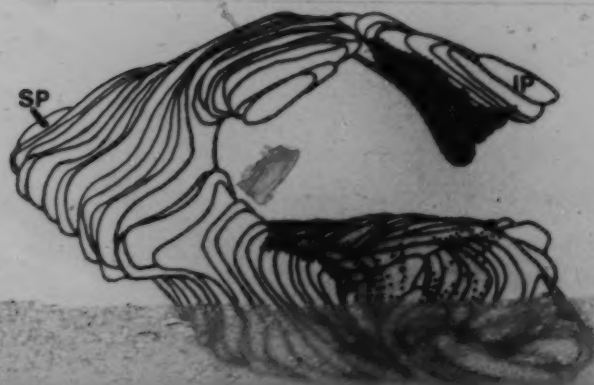
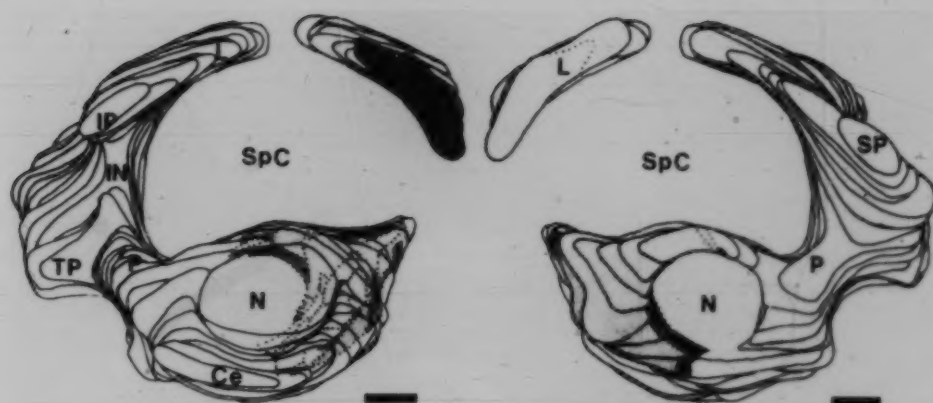


Figure 41. Serial reconstructions of C10 of embryo 52/2 (stage 30)

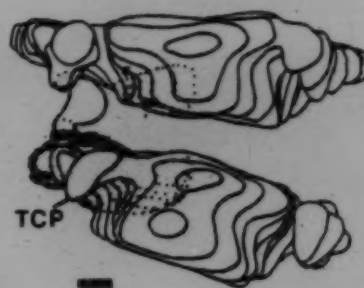
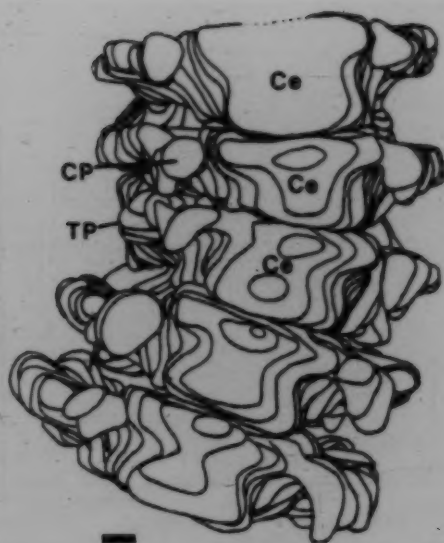
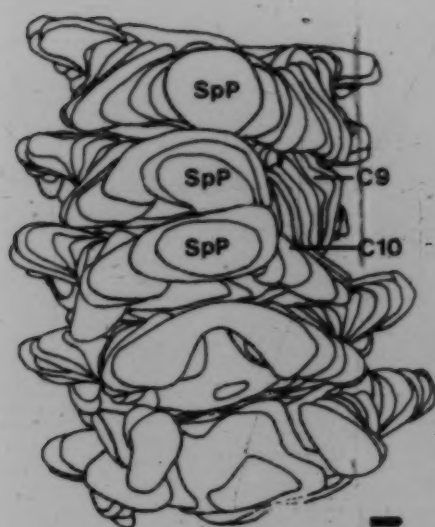
Caudal (above left), rostral (above right), and caudal separated views are presented. The pedicle and transverse process of C11 are fused to the caudal aspect of the centrum. An intervertebral foramen partially separates the fused arches.

Bar = 100 μ m



Figure 42. Serial reconstructions of C8 to C12 of embryo 96/5 (stage 32)

Dorsal (above left) and ventral (above right) views of the two quail-containing vertebrae and adjacent vertebrae. The quail transverse process is less extensive than adjacent ones. Below, the quail region has been shaded in. The continuity of the quail region across the two vertebrae is apparent. Lower right is a partial reconstruction with the two vertebrae partially separated to illustrate the vertebral boundaries and the quail content of each. Bar = 100 μ m



Embryo 75/3 seemed to represent the typical quail cell distribution in the vertebrae. From the reconstructions it could be seen that the quail cells occupied a significant proportion of the caudal neural arch (Figure 37) and a relatively smaller portion of the rostral neural arch (Figure 38). Quail cells were present in almost equal volumes of the two centra. The continuity of the quail cell area across the two vertebrae was apparent in the composite reconstruction of C5 and C6 (Figure 39).

Several distinctions between the two sides of each of the vertebrae in embryo 75/3 were apparent from the composite drawings:

1. The chick side of the spinal canal appeared more rounded, whereas the quail side was somewhat triangular.
2. The dorsoventral extent of the quail portion ~~of the centrum~~ was smaller than the contralateral chick side.
3. The quail costal process was less extensive than that of the chick.
4. The transverse foramen was situated more medially on the quail side than on the chick side.
5. The superior and inferior articular processes were positioned more ventrally on the quail side than on the chick side.

In the sections, it was often apparent that the quail disc was much thinner rostrocaudally than the chick counterpart.

In the reconstructions of the vertebrae of embryo 52/2 (stage 30) a similar pattern of quail cell distribution was noted (Figures 40.

also displayed several abnormalities. The pedicle and transverse process of C11 were joined to the caudal portion of the C10 centrum. An intervertebral foramen partially separated the two pedicles. However, the remainder of the two neural arches were fused. In the sections a spinal ganglion could be noted in the intervertebral foramen.

A longitudinal view has been presented in Figure 42. Embryo 96/5 (stage 32) again displayed a similar pattern of quail cell distribution. Quail cells were present in adjacent quadrants of the two centra, a large portion of the caudal neural arch, and a relatively small part of the rostral neural arch. Some quail cells were present in the inferior tip of the costal process of the rostral vertebra. A few abnormalities were also apparent in the two vertebrae.

v. Summary of Late Phase

A number of features were distinctive in this group of embryos.

1. Quail cells occupied positions in two adjacent vertebrae in all embryos.
2. The centra, laminae, and apposing articular processes demonstrated quail cell continuity across the two levels fairly consistently.
3. The pattern of cellular contribution to the pedicles, transverse processes, costal processes, and spinous processes was unclear, although certain tendencies were apparent.

5. Differences in the distribution of the various quail somite derivatives were observed.
6. The occurrence of spinal ganglion abnormalities was closely related to the presence of neural arch defects.
7. Presence of abnormalities coincided with lack of correspondence between the somites in three cases.

5. General Summary of Results

Some generalizations could be drawn based upon the pattern of quail cell distribution during the developmental period examined.

1. In successively older embryos there was a progressive increase in the number of quail cells observed.
2. Quail cells were found forming a variety of tissues, including cartilage, muscle, meninges, and connective tissue.
3. Quail cells initially remained within delineated boundaries, but in older stages there was more mixing of chick and quail cells, particularly in muscle and connective tissue.
4. In the vertebral rudiments the quail cells were more localized with minimal intermingling of the two cell species.
5. Some shifting of the cellular condensation patterns was apparent in successive stages of the embryos.
6. A variety of abnormalities in the vertebral elements was observed, most of these being associated with anomalies of the spinal ganglion or lack of correspondence between the host and donor somites.
7. In a number of embryos, a significant portion of the quail cell

vertebral region was populated by chick cells, noticeably in the perinotochordal and perineural areas.

8. In the older embryos a distinct pattern of quail cell distribution could be distinguished, quail cells being present in portions of two adjacent vertebrae in all cases.
9. Generally, the quail cells did not extend past the superior border of the inferior notch in the more rostral of the two vertebrae.

V. DISCUSSION

1. Somitic Contribution to Vertebrae

The basic questions which were raised in the introduction have been answered by this study, within the limitations of the methodologies employed.

1. What is the cellular contribution of a single somite to the vertebral column? It has been demonstrated that cells from a single somite contributed to the formation of two consecutive vertebrae, in agreement with the resegmentation theory proposed by Remak (1855; cited in Verbout, 1976) and supported by a number of other investigators, including Piiper (1928), Williams (1942), and Sensenig (1949). The distribution of the cells from a single somite, however, was not equal between the two vertebrae in all of the vertebral elements.

2. What are the relationships of sclerotome cells from a single somite to adjacent structures during the early phases of vertebral formation? Sclerotome cells were seen to remain largely within the initially defined boundaries of the intersomitic vessels, occupying axial regions primarily on the side ipsilateral to the original somite.

A. Vertebral Elements

i. Centra and Disc

The quail cell content of the notochord-associated structures indicated that the intervertebral disc formed centrally in the medial sclerotome from a single somite. The cells rostral and caudal to the disc region merged with cells from the adjacent somites to form the rostral and caudal centra. Each centrum, then, must be a composite of cells from two consecutive somite pairs, each pair making approximately equal contributions, and the disc forming bilaterally within one somite pair.

ii. Laminae

Quail cells were always found in the adjacent laminae of the same two quail cell-containing centra. As could be seen in the serial reconstructions of embryos 75/3 (Figures 37 to 39) and 52/2 (Figures 40, 41) the quail cell region extended only a short distance into the more rostral lamina. In particular, quail cells were found in only a few sections (approximately 100 μ m or less) rostral to the superior border of the inferior notch. As this was the original rostral boundary of the somite (position of the spinal ganglion) most likely the normal contribution to the rostral lamina would be relatively small. Conversely, the single somite contribution to the caudal lamina should be relatively large. If, in the two embryos for which transverse serial reconstructions were presented, the areas abnormally fused to the rostral vertebra were added instead to the caudal vertebra

almost that entire caudal lamina would have been composed of quail cells. Cells from a single somite, then, normally should form parts of two adjacent laminae, but a far greater portion of the more caudal lamina.

iii. Spinous Processes

Somitic contribution to the spinous process should be closely associated with that of the lamina, as each spinous process seemed to be an extension and fusion of the two bilateral laminae. Quail cells were found in the two spinous processes (or precursors) of adjacent vertebrae in 10 of 20 embryos. The cases in which neither spinous process contained quail cells have been discounted as having likely resulted from lack of complete integration of quail cells. The pattern of cellular contribution from a single somite in the two spinous processes probably should correspond with that of the two laminae: a large proportion of the caudal spinous process and a relatively smaller proportion of the rostral spinous process.

iv. Articular Processes

Somitic contribution to these processes also showed a very distinct pattern, with quail cells located in the adjacent articular processes in 18 of 20 embryos. The continuity of the cell distribution across the facet joint was apparent from the composite reconstruction of both vertebrae of embryo 75/3 (Figure 39). As the 2 embryos which deviated from this pattern displayed either numerous abnormalities, or showed poor integration of quail cells, it was decided that they

demonstrated atypical quail cell distribution. It would appear that adjacent articular processes, as well as all the tissues which form the joint surfaces and joint capsule, should be formed of cells from a single somite.

v. Pedicles

In contrast to the previously described elements, the quail cell contribution to the pedicles was somewhat more difficult to interpret with so many abnormalities present. Excluding embryos which demonstrated extreme anomalies (fusions or absence of the caudal pedicle), quail cell content was the more significant in the caudal pedicle. It is suggested that the contribution of cells from a single somite would be to the formation of most of the caudal pedicle and of the base of the rostral pedicle (dorsal extension of the centrum) as was depicted for embryo 75/3 (Figures 37 and 39). Somitic contribution to the pedicle normally would not extend past the rostral border of the inferior notch of the rostral vertebra, consistent with the pattern seen in other parts of the neural arch.

vi. Transverse Processes

In the transverse processes, again the majority of 'normal' cases suggested that quail cell contribution was more substantial in the caudal transverse process, and minimal if at all in the rostral transverse process (12 cases). In most of the remaining embryos, various abnormalities and/or unusual quail cell distribution in other vertebral areas were present. It was noted that the quail cell

distribution in the transverse processes frequently matched that of the pedicles, possibly indicative of developmental synchrony in these two elements.

vii. Costal Processes

Similar to the observations made for the transverse processes, quail cell distribution in the costal processes was more suggestive than distinct. In 12 embryos the caudal costal process was almost entirely made up of quail cells and only a small number of quail cells were found in the inferior tip of the rostral costal process. The complementary location of chick cells in the inferior tip of the caudal costal process further supported the proposed imbalance of cellular contribution from a single somite to consecutive costal processes.

B. Summary

From these observations it was apparent that cells from a single somite took part in the formation of two adjacent vertebrae. Each vertebra then would seem to be a composite of cells from two initially distinct populations: two consecutive pairs of somites. The half-centra were formed of approximately equal portions of chick and quail cells, the intervening disc being entirely quail. In the neural arch, a somewhat different pattern of distribution was indicated. Although the majority of somitic contribution was to the caudal arch, some was also made to the inferior articular process of the rostral vertebra, and to small portions of the other elements, up to the transverse plane

of the superior border of the inferior notch. The proposed pattern of somite dispersal has been illustrated in Figure 43.

2. Limitations Inherent in Methodology

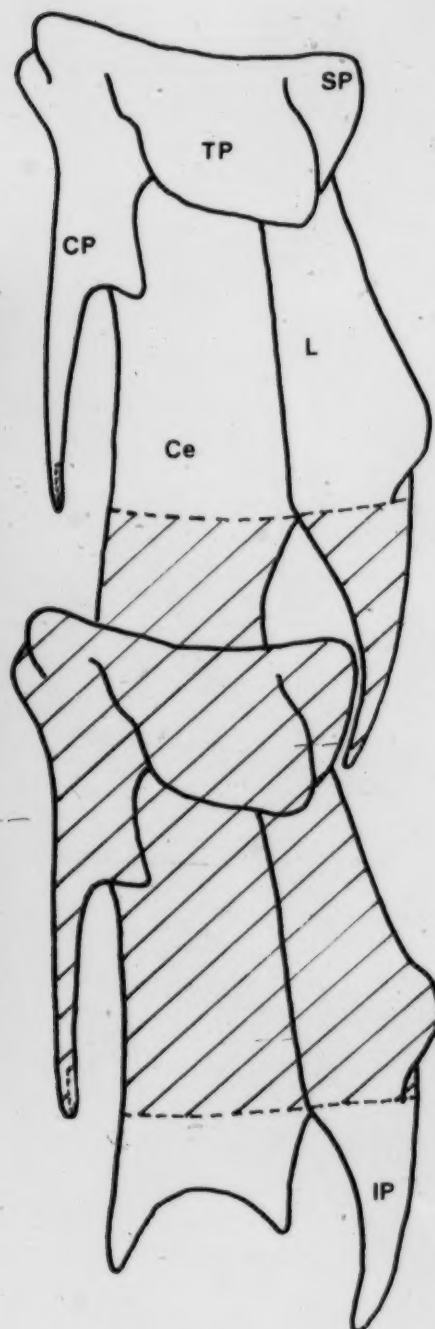
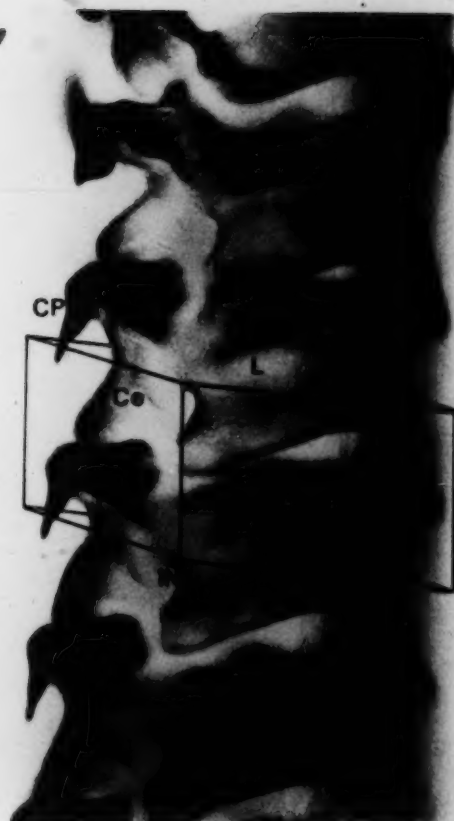
Although fairly consistent cellular distribution patterns were observed in certain of the vertebral elements, a number of considerations must be kept in mind when interpreting these results. Due to the methodology employed in this study, several variables were introduced which may possibly have obscured the actual single somite contribution to the vertebrae. The possible effects of these variables require further discussion.

A. Host Embryos

As a result of the normal variations in embryonic stage when the eggs were first obtained, and in individual developmental rate, a wide range of stages were observed at the time of windowing. To stay within the appropriate phase for the extracted somite, several different levels of cervical somites were removed. Consequently, it was necessary to assume that vertebral morphogenesis was the same at all the levels studied within the cervical region. It is probable that any differences which may exist would be related primarily to differences in the shape of the definitive vertebra, as suggested by the results of the work by Kieny *et al.* (1972) on regional determination of somites. As the mid-cervical vertebrae are morphologically very similar, it is suggested that the assumption was most likely valid.

Figure 43. Proposed contribution of cells from a single somite to formation of two adjacent vertebrae

Mid-cervical vertebrae from a 10-day chick embryo are shown on the left. On the right is a representation of mature ossified mid-cervical chicken vertebrae. Note that the vertebral region formed by cells from one somite crosses two adjacent centra. The limits of single somite contribution are formed by the inferior notches of the pedicles. Also note the contribution to the inferior tip of the costal process.



For all of the host embryos, thoroughness of somite removal could not be ascertained. Trials designed to examine by SEM the efficiency of somite removal demonstrated that, although in many cases the somite space had appeared clean at the time of removal, frequently some small fragments remained (Figure 7). Any cells of the chick somite which were inadvertently left behind would likely have developed in synchrony with adjacent chick somitic cells further limiting the opportunity for normal progression of the implanted quail somite. This situation possibly occurred in many of the embryos in which a large proportion of chick cells populated the quail cell region, and might explain some of the abnormalities and variation of quail cell integration that were observed (examples in Figures 14, 15). The difficulty encountered in attempting to determine completeness of somite removal could not be resolved. Kieny *et al.* (1972) indicated, in fact, that they made no attempt to remove the ventral portion of the somites in their study, to avoid possible injury to underlying structures.

The inability to account for all of the host somitic tissue was possibly one of the major limitations with respect to analysis of the quail cell dispersal patterns observed. The assumption had to be made that normal quail cell integration was present only in embryos in which the quail region extended from the ectoderm to the axial organs, and completely filled one segment.

As was suggested by the work of Strudel (1967), possible damage to either the notochord or neural tube during somite removal may have resulted in some of the abnormalities observed (examples in Figure 36). Based upon the studies by Gallera (1966) and Strudel (1966) it is also

possible that disruption of the adjacent epithelial layers may have adversely affected normal progression of the implanted somite. Although fairly rapid healing of chick ectoderm (and so probably endoderm) following deliberate insult has been demonstrated (Stanisstreet *et al.*, 1980), the phase of the somite during the particular stage selected for somite removal would appear to be a critical one with regard to the cellular interactions occurring that lead to successive developmental events. Damage to the two epithelia (of necessity for the ectoderm; accidentally in the case of the endoderm) may have disturbed the normal sequence in development resulting in some of the unusual dispersal patterns which were observed (example in Figure 14b, embryo 40/2).

B. Donor Somites

Difficulties encountered with the donor somite often produced a mismatch with the host somite number, and/or a lack of synchrony in somite maturity. Some of the abnormalities observed may have resulted from the effects of these two factors, as suggested by the results of Kieny *et al.* (1972) regarding regional specification of sclerotome cells (possible example in Figure 15, embryo 29/6).

Lack of adequate cleaning of the donor quail somite was apparent in 4 embryos in which some sections showed quail nephric ducts present within the somitic cell region. A small piece of the intermediate mesoderm possibly remained attached to the implanted donor somite and formed the isolated tubules. The displaced chick nephric ducts which were observed in 2 embryos suggested that during somite removal the

intermediate mesoderm had likely been damaged and a small portion of it had fallen into the somite space.

C. Transplantation

Removal of a somite from the host embryo usually resulted in the two adjacent somites bulging into the space created (also reported by Packard and Jacobson, 1979). Similarly, an isolated quail somite seemed to expand in size and became more spherical in shape when not confined by surrounding structures. The transplant procedure then involved inserting a 'larger-than-normal' somite into a 'smaller-than-normal' somite space. Some of the variability of quail cell integration noted may have resulted from an inability to maneuver the single somite into the plane of the host somite column. A possible example of this situation was shown in Figure 14a in which the quail somite had remained as an intact epithelial ball, lying just deep to the ectoderm.

Experiments involving single somite removals without replacement have shown that at least some cell regulation does occur in an apparent attempt to replace the excised tissue (Strudel, 1966; Kieny *et al.*, 1972; Bagnall *et al.*, 1986). If the inserted quail somite did not adequately fit the space, the process of regulation was likely initiated and chick cells from adjacent regions may have moved into the area. This would be particularly true in the case of the ventral and ventromedial regions of the somite space as compression forces would tend to displace the donor somite dorsally through the defect in the

ectoderm. Many examples were seen in which chick cells were occupying ventromedial positions in the quail region (Figures 14, 15, 22).

The process of cell regulation in somitic mesenchyme is a poorly understood phenomenon. Although Strudel (1966) has claimed that complete regulation occurred following removal of up to 5 somites, Kieny *et al.* (1972) observed that some vertebral abnormalities developed when they excised somites or segmental plate mesoderm. Recently, Bagnall *et al.* (1987) have demonstrated a range of somitic regeneration following single somite removals. It may be that cell regulation in somites is an unstable and highly variable event, possibly dependent upon factors which are as yet undetermined.

One of the major causes of the vertebral and sclerotome abnormalities was probably related to incorrect orientation of the implanted somite. Experiments by Keynes and Stern (1984) and by Rickmann *et al.* (1985) showed that spinal nerve fibers and neural crest cells are apparently able to distinguish between the two sclerotome halves, growing or migrating preferentially only into the rostral half. The distinction between the cells in the two sclerotome populations apparently occurs prior to somite segmentation and therefore presumably was present in the donor somites of this study. The findings from both of these studies have implications concerning vertebral morphogenesis and segmentation. Investigations by Detwiler (1934) and by Strudel (1967) have indicated that the ventral nerve roots and the spinal ganglia (the latter being derived from neural crest cells) influence segmentation of the centra and the neural arches, respectively. The potential problems which may have arisen as a result of inappropriate

orientation of the somite would seem to be somewhat complex; these have been discussed further in following sections.

The effects of the intrusion itself into the host embryo were not known. The apparent complexities of the continuing cellular interactions are only recently being appreciated, but still are not well understood. Further primary limitations of this study, then, were the necessary assumptions that:

1. invasion of the host tissue, removal of a somite, and replacement of that somite with a somite from another animal did not interrupt normal development of the host; and,
2. the implanted foreign tissue responded in the same manner as the extracted host tissue would have behaved.

Since severe abnormalities were observed in the homospecific transplant embryos as well as in the heterospecific transplant embryos, non-integration potentially due to species differences was determined to have been minimal. The validity of the former assumption, on the other hand, may be questionable.

The possibility that differing developmental rates inherent in the two cell species may have affected the observed results must be considered. It has been implied by Chevallier *et al.* (1977) and by Erickson *et al.* (1980) that quail cells may mature more rapidly than chick cells, thus populating developing organs earlier. The number of embryos in this study which displayed poor quail cell integration would tend to suggest that the effects of potential differences in developmental rates may have been less than anticipated.

Numerous investigators have employed techniques involving replacement of host somites or portions with comparable tissue from another embryo of the same or different species (Detwiler, 1934; Hazelton, 1970; Kieny et al., 1972; Chevallier et al., 1977; Christ et al., 1977; Beresford, 1983; Noden, 1983; Stern and Keynes, 1987). In all cases, a normal sequence of development had to be assumed to permit tracing of the cell population under investigation. As no method has yet been developed to distinguish cells descendent from a particular somite *in situ*, utilization of interspecific transplants which permit cell species identification would seem to be the best alternative.

D. Analysis

Despite Le Douarin's (1985) claim that a single quail cell could be identified within a chick cell population, it was concluded that in a given section, some cells could not be species-typed with absolute certainty. As was noted in Chapter IV (Results), some chick cells were observed to have very large darkly stained nucleoli and may have occasionally been mistaken for quail cells. It was also possible that quail cells in which the nucleoli were absent from or only partially within the plane of a given section may have been presumed to be chick cells (Figures 9, 10). The proportion of false positive and false negative quail cell identification seemed to be quite low, but some error in delineating quail cell regions, particularly boundaries, probably occurred. This potential error was determined to be acceptable within the scope and the objectives of the study as the error was assumed to be primarily in the identification of isolated

cells, minimally affecting valid delineation of large regions of quail cells.

Since no external references were inserted into the embryos or embedding medium prior to sectioning, some error was probably introduced when generating the serial reconstructions. For the embryos which were sectioned transversely, the features which were assumed to be constant (notochord and central canal of the spinal cord) likely did shift to some degree relative to the vertebra. In the embryos which had been sectioned longitudinally the method of "best fit" employed was less than ideal, but did afford an apparently adequate representation of the quail cell distribution from a different perspective. Although there may have been some distortion of the vertebrae in regard to accuracy of the reconstructions, the potential error was determined to be of a relatively minor consequence as the shapes of the primary vertebral elements were within acceptable limits.

The serial reconstructions were produced to afford an alternative means of presenting the typical quail cell distribution. It should be appreciated that a three-dimensional configuration could not be presented accurately in two dimensions using this methodology; factors such as perspective and depth were not accounted for. Additionally, the separated reconstructions (e.g. Figure 39) did not represent rotated views of the vertebrae, but showed only slightly separated individual sections as they would each have appeared from a direct caudal view. Limitations, then, were imposed regarding the inability to observe each vertebra and its quail cell content from different perspectives. It is suggested that reconstructions of embryos that had

been sectioned in different planes, as was done in this study, partially circumvented this problem.

3. Vertebral Formation

It was not within the scope of this study to provide a detailed account of the various events that occurred during vertebral development. This topic has been adequately described in a number of works: Piiper (1928), Williams (1942), Sensenig (1949), Verbout (1985). Some features of this process, however, were notable in that cells from a single somite could be identified, unlike previous studies of vertebral formation which did not utilize a permanent cell-marker system. It was believed that some new information, based upon the methodology employed, could be provided. Certain of the observations, then, warrant further discussion.

The most apparent conclusion to be derived was affirmation of the somitic origin of the vertebrae: centrum, intervertebral disc, and neural arch. Although the suggestion from past studies that extraembryonic cells formed the vertebrae (His, 1868: cited in Verbout, 1976) has generally been discounted, some recent investigators have distinguished between the perichordal tube and lateral sclerotome densities, with the somite as the probable origin of the perichordal tube cells (Dalgleish, 1985; Verbout, 1985). Verbout (1985), in fact, suggested a possible angiogenic origin of the cells forming the perichordal tube. This study has demonstrated that both the lateral sclerotome and the perichordal tube were comprised of somite-derived

cells. The developmental progression of each of these two regions, however, may involve separate processes.

Formation of the centrum and neural arch would seem to have occurred independently, as was apparent from the abnormalities in which a centrum developed in isolation with its neural arch fused to the next vertebra (Figures 40 and 41, for example). Strudel (1953: cited in Strudel, 1967) has demonstrated that rudiments of either of these two vertebral portions could form in the absence of the inductive agent (notochord or neural tube) for the other vertebral portion. The factors involved which result in the union of these two parts of a vertebra may be related to intrinsic mechanisms, or to chondrification processes.

A number of studies have suggested various locations for the intervertebral disc within the cell population from a single sclerotome, with correspondingly different cellular contributions to the rostral and caudal centra. In this study, the disc was seen to form in the middle (rostrocaudally) of the quail region, with approximately equal quail cell distribution in the two adjacent centra. Whether it may have formed at the interface of the two sclerotome subpopulations or entirely within one of the two regions could not be determined.

Another feature of interest was the observation in many embryos of chick cells and quail cells forming opposite (rostral-caudal) surfaces in portions of the lateral densities (Figures 20b, 27). These condensations were the developing transverse and costal processes. The dual composition of these processes may have reflected the possibly

variable, although minor, contribution of the rostral portion of the sclerotome to the rostral transverse and costal processes, and the fairly consistent involvement in forming the tip of the rostral costal process (Figures 38, 42). It may also have represented the connective tissue (tendon) attachment of the intervertebral muscle to the rostral process. The muscle which spanned the gap between rostral and caudal lateral processes was almost entirely quail, occasionally with a few chick cells possibly present. Some sections through costal processes clearly depicted the situation of an entirely quail-celled process next to an entirely chick-celled process with quail muscle intervening (Figure 32). This observation has definite implications with respect to one of the basic premises of the resegmentation theory (discussed below).

A. Resegmentation

Although some variation appeared to exist in the literature in regard to different authors' interpretation and application of the term "resegmentation", there would appear to be a fairly common ground from which to proceed. It was assumed by this investigator that the intended meaning was that one definitive vertebral unit or segment (vertebra and the caudal intervertebral disc) was formed of cells derived from two consecutive pairs of previously existing units or segments, the somites. On this basis, the present study has demonstrated that resegmentation did occur, confirming Remak's (1855: cited in Verbout, 1976) original hypothesis. Arguments, then, as to whether or not physical separation of the cell populations in the

interim stages were present and later replaced by new ones (i.e. intersomitic clefts and intrasclerotomic fissures) would seem to be irrelevant. Vertebrae were demonstrated to be of dual somitic origin regardless of whether the clefts and fissures were present, as demonstrated by Keynes and Stern (1986), or artifactual, as indicated by Dalglish (1985) and Verbout (1985). It would seem likely, however, that the possible presence of these boundaries may reflect patterning mechanisms resulting in the eventual segmentation of the vertebral column (discussed further in Pattern Formation).

Among the bases of the arguments supporting resegmentation were the assumptions that the sclerotome cells from a single somite retained their original positions relative to the intersomitic blood vessels and to the dorsal ganglion. These assumptions were largely hypothetical as the investigators had no means of identifying the original cell population. This study demonstrated, however, that the chondrogenic cells of the sclerotome did remain approximately within the initial boundaries of the intersegmental vessels, and that the spinal ganglion continued to approximate the rostral border of the sclerotome throughout the period examined. An additional feature of the resegmentation theory was resolution of the alternation of muscle and vertebrae such that the contractile tissue was positioned across the joint. A significant observation of this study was quail or chick muscle apparently attached to either quail or chick cartilage. It would seem that resegmentation is not a prerequisite for development of a functional system: muscle could attach to cartilage derived either from its own original segment, or from the adjacent segment.

The various connotations of the term "segment" have presented some confusion in the literature. For example, it was unclear as to whether the term segment should be used to refer to a single somite, the sclerotome from a single somite, or only the lateral portion of the sclerotome from a single somite (perichordal tube material excluded). The most obvious original segments were the somites which, while still intact, had clearly defined boundaries. The axial segments of the mature animal, however, were the vertebrae and discs. From a developmental standpoint, the definition of an original segment should be the region from which is derived the cells that eventually form the vertebral segment: the caudal and rostral sclerotome halves of two adjacent somites and the intersomitic cleft located centrally. The possible existence of the intrasclerotomic fissure (Stern and Keynes, 1986) would further support this alternative definition of a segment, although it is a feature non-essential to the concept.

4. Cell Dispersal Patterns

As was seen in the earliest stages of sclerotome dispersal the somitic cells remained within a delimited region. The number of quail cells observed outside the major quail region increased in successively older embryos, such that by stage 36 there was substantial mixing of the two cell populations. The amount of interspecies cell mixing, however, was not equivalent among all the somitic derivatives.

A. Chondrogenic Cells

The quail cells which formed the vertebrae remained generally within the region that had been earlier bounded by the intersomitic vessels, although a few isolated exceptions were seen. Quail cells in the vertebra were occasionally observed to have crossed the mid-sagittal plane to the contralateral side, usually in association with the notochord having been displaced in the same direction. Additionally, a minimal amount of mixing with ipsilateral chick-celled regions occurred in embryos exhibiting more complete quail cell integration.

B. Meningeal Cells

The relative shift in the positions of the vertebral quail zone and the meningeal quail zone may have been produced by caudal migration of the meningeal quail cells. It was also possible that the discrepancy in position resulted from a differential in rostrocaudal growth rates of the vertebral column and the neural tube. The vertebral precursor cells were observed to remain at approximately the same level with respect to the intersomitic vessels, whereas the quail meningeal region was shifted slightly caudally. If the meningeal quail cells became intimately associated with the surface of the neural tube then they would necessarily be displaced with that surface during growth in length of this structure.

C. Myogenic Cells

Quail cells were frequently seen mixed with chick cells in the axial muscles at some distance from the quail vertebral region. It was not known, however, whether the invading cells were derived from the dermamyotome, or if they represented an additional subpopulation of the somitic mesenchyme. It has previously been assumed that voluntary musculature formed entirely from the myotome portion of the somite. During development the myotome remained as an intact sheet of cells which progressively extended dorsally and ventrally to form the epaxial and hypaxial muscles. Studies on limb musculature formation have concluded that single mesenchyme-like cells disassociated from the ventrolateral border of the still intact somite and migrated to the prospective limb bud region, later to form the intrinsic limb muscles (Chevallier *et al.*, 1977; Christ *et al.*, 1977). Observations of individual cells detaching from the somite or the myotome and migrating to become incorporated into other myotomes, however, have not been reported. Although the specific origin of the displaced muscle cells could not be determined, the possibility that they may have come from the somitic mesenchyme could not be denied.

5. Pattern Formation

As has been demonstrated in studies of several systems, there are potentially a number of factors which may be involved in cell patterning, and so could contribute to the shaping of the definitive structure. Each of the cells within a developing system is confronted with possible influences from its surrounding environment, in addition

to being directed by various intrinsic mechanisms. Among these cellular interactions and processes some have been shown by previous investigators to be applicable to vertebral formation.

The mature vertebral column is distinctive in that it consists of a repetitive series of similar individual units. A single vertebra, however, is somewhat complex in shape, possessing a number of irregular surface features. Although many of the steps involved in the process of chondrification have been established, stages which lead to morphogenesis of a definitive vertebra have been less extensively investigated. In particular, one of the least well understood aspects of vertebral development is the sequence of events involved in formation of individual units within the column.

A. Segmentation

Strudel (1967, and cited in Hall, 1977) has indicated that segmented centra developed only in the presence of a normal notochord and ventral nerve roots. It was possible that, in the present study, abnormal fusion of adjacent centra may have resulted from either injury to the notochord, or inappropriate location or absence of motor nerve roots. Probable random orientation of the somite, when considered in conjunction with preferential growth of motor neurons into the rostral sclerotome half (as determined by Keynes and Stern, 1984) may have been the underlying cause of the centrum anomalies which were observed. It then becomes difficult, however, to account for the number of instances in which relatively normal segmented centra or precursors had developed. Possibly the system may require appropriate cellular

interactions within the perichordal tube as well as inductive influences from the notochord and the ventral nerve roots.

Separation of the neural arches, on the other hand, has been shown to be dependent upon the presence of spinal ganglia (Detwiler, 1934; Williams, 1942; Strudel, 1967). In accord with this dependency, observations in the present investigation demonstrated that unusual location of the spinal ganglion was frequently associated with abnormal fusion of parts of the neural arch. As explanations of this possible cause-effect relationship, Flint (1977) suggested that the migrating neural crest cells mechanically displaced the sclerotome from the rostral region, resulting in cellular compaction of the caudal region. It could be seen, in early morphogenesis, that the ganglia physically separated adjacent neural arch rudiments and so could produce segmentation merely by their location. The interactions which would lead to separation of the later developing dorsal extensions (laminae and spinous processes) and lateral processes, however, are less clear.

In the older embryos of this study, it could be seen that some chondrified elements were located in the same transverse plane as the spinal ganglia: there was no physical separation of the processes by an intervening structure. Additionally, there were some cases in which a ganglion was present and neural arches had fused around it (Figure 41). It may be that ganglia somehow inhibit chondrogenesis of cells in the immediate vicinity early in development and so influence later patterns of chondrification. As well, characteristics such as appropriate size and shape of the ganglion could be necessary for normal separation of the arches to occur. Chondrifying elements which

are abnormally close to each other may simply fuse together producing some of the unusual patterns that were observed. It would seem that there are factors additional to the mere presence of a spinal ganglion involved in producing normally segmented neural arches.

B. Induction and Cell Determination

Although this study did not trace the various cell differentiation pathways, some of the observations could be related to current knowledge of somitic cell determination. One of the more significant conclusions to be derived from this investigation was the possible pluripotent nature of the somitic cells. Since orientation of the transplanted somite was undetermined, it could be assumed that it was most likely random, with a wide range of orientations obtained. This assumption would seem to be valid when combined with the observation of varied locations of the spinal ganglion within the quail cell region, and the findings of Rickmann *et al.* (1985) regarding neural crest cells migratory pathways. Unusual orientation of the somite, then, would place cells which had possibly been predetermined for a particular fate into a new local environment. In many instances the new local environment was apparently able to redirect the development of the cells.

Stern and Keynes (1986) have demonstrated that determination of sclerotomal cells as either rostral or caudal occurred prior to somite segmentation, and that this determination was irreversible. Additionally, Rickmann *et al.* (1985) observed that neural crest cells migrated only into the rostral half of the sclerotome. In agreement

with the findings of these two studies, several instances were noted in this investigation in which the ganglion in the quail cell region was fused with the next caudal ganglion, or the ganglion within the quail region was very small or absent and the next caudal ganglion very large. If the orientation of the donor somite had been inverted about its medio-lateral axis, two rostral sclerotome halves would have abutted each other directly. Assuming that the neural crest cells migrated only into the rostral sclerotome half, and that no cell-sorting occurred, a large ganglion would then form on the boundary of the two somites. Some examples were seen in which the quail cell region bounded the rostral half of an abnormally large spinal ganglion (Figure 21). It would seem that the cells were unable to reorganize into appropriate locations, or to redifferentiate with respect to the new orientation of the somite. This conclusion has implications regarding vertebral morphogenesis, as discussed previously.

The inductive influence of the ectoderm and endoderm on formation of the dermamyotome and sclerotome respectively, has been demonstrated by Gallera (1966). In this study, the cells of the transplanted donor somite would have been associated with these epithelia in the quail embryo prior to removal, yet the donor cells formed relatively normal derivatives in the host embryo in most cases. As well, the potential predetermination of somitic cells as either chondrogenic (Hall, 1977, 1986) or myogenic (Wachtler *et al.*, 1982) would also appear to be reversible, as has been demonstrated in previous studies (Holtzer and Detwiler, 1953; Strudel, 1953: cited in Hall, 1977). The sections of the older embryos in this investigation generally showed cartilage

centrally, dermal connective tissue peripherally, and muscle situated between the two. It could be argued that the cells underwent extensive migration following the transplant to reorganize into their original locations; this possibility would seem improbable as a normal-appearing quail dermatome and sclerotome were usually present within 24 hours of the operation.

Speculation could be made as to whether or not somitic cells were pre-determined to form specific vertebral elements. This specification likely did not exist for the notochordal structures since relatively normal centra and intervertebral discs were observed in many cases in this study. The situation for the neural arch and, in particular its various processes, however, was not as clear. A variety of abnormalities were observed in the arches including the presence of anomalous pieces of cartilage. If some regions of cells were irreversibly determined to give rise to particular vertebral processes one might anticipate the abnormal results which were observed. It is suggested, however, that alteration of the local environment may have been the factor primarily responsible for the various anomalies which developed. In particular, more severe abnormalities were observed in the embryos in which a large proportion of chick cells were present within the quail cell region. Potential interaction between remnant chick tissue or additional chick tissue from regulation processes, and derivatives of the implanted quail somite may have resulted in some of the unusually-shaped elements.

C. Genetic Limitations

Investigation of vertebral formation in allophenic mice showed that some vertebral characteristics in the chimeric progeny could be identified as particular to one or the other of the two strains of mouse used in the study (Moore and Mintz, 1972). Additionally, heterospecific somite transplants in *Ambystoma* embryos resulted in formation of neural arches which were of the donor type (Detwiler, 1938). It was probable, in these cases, that genetic determinants imposed restrictions on the shape of the vertebrae formed from the implanted cells. Several examples were noted in this study in which some properties of the chimeric vertebrae appeared to be characteristic of quail vertebrae (Figures 37 and 38).

The question as to the classification of vertebral parts as either chick or quail could not be answered by this study. Accurate determination of the species-type of vertebral elements could be accomplished only through a detailed morphometric analysis. Some trends were apparent, however, which suggested that genetic limitations might have been imposed upon the quail cells. This may have been one of the contributing factors which resulted in the development of atypical vertebrae in the host embryos. It was likely that the chick environment acted as a modifier to some extent such that actual quail vertebral elements did not develop.

6. Summary and Implications for Further Study

Based upon the results of this study, it would seem that resegmentation of the descendents from the original segments (somites) in formation of the mature segments (vertebrae) did occur. The apparent realignment of tissue may have resulted from intrinsic mechanisms of the cells themselves, from influences of the local environment, or from a combination of the two. It is suggested that the events which lead to segmentation of the vertebral column warrant further investigation.

The cells derived from a single somite would seem to comprise a number of subpopulations, each exhibiting distinctive characteristics. In particular, a wide range of dispersal patterns among these subpopulations was observed in this study. Consideration of the cellular diversity of somitic descendents then, should be considered when examining behavioral properties of somite cells or when studying morphogenesis of the various systems to which somites contribute.

In this study, it was not possible to determine if normal development of the axial structures would have ensued had all factors been accounted for. The difficulty encountered in maintaining appropriate orientation of the single somite suggested that a preferred method would have been to replace two consecutive chick somites with the corresponding pair of quail somites. Correct rostrocaudal orientation could then at least have been ensured; orientation in the other two cardinal planes might have been achieved through the use of carbon particle markers.

Significant implications arise from this investigation regarding the events which are involved in vertebral morphogenesis. Potential interactions between developing systems, mechanisms involved in segmentation, cell specification in forming particular vertebral elements, and chondrification patterning are among several areas which present additional questions to be answered in future investigations.

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APPENDIX A

PRELIMINARY EXPERIMENTS PERFORMED

In addition to the actual transplants, a number of experiments were performed to determine the most effective method of visualizing the almost-transparent chicken embryos *in ovo*, and to examine other factors which could possibly influence results. A series of tests were performed to determine the effects (if any) of the experimental procedures on survival rate of the embryos. Items examined included: candling of the egg, wiping the shell with 70% ethanol, removing 1.0 ml albumen from the egg, windowing the egg, adding Pannett and Compton's saline with antibiotics to the embryo, and operator wearing a gauze mask during the operations.

One of the methods employed to render the chick embryos visible *in ovo* was application of one or two drops of a 0.1% solution of neutral red in Pannett and Compton's saline onto the vitelline membrane immediately over the location of the embryo. Forty-one trials were performed on embryos between stages 9 and 14 (Hamburger and Hamilton, 1951) to determine the effect of neutral red on embryo development. A similar series using 8 two-day embryos was performed using weaker concentrations of neutral red (0.01% to 0.075%). Nine trials were performed in which one or two drops of the 0.1% neutral red solution were dropped on the vitelline membrane; the membrane was then torn and each egg reincubated. The embryos in each case were examined every one or two days to discard any dead or severely abnormal ones. Evaluation of the trials was made on the eighth day.

To examine different methods of neutral red application, a 2% agar mixture was prepared using 0.1% neutral red solution. A few drops of the mixture were spread onto microscope slides to leave a thin film on the surface. The slides were placed in the refrigerator until the mixture had hardened. When required, a slide was removed and some of the agar scraped off the slide using a razor blade. A small flake of agar was placed on the vitelline membrane overlying the chick embryo for 2 to 5 seconds and then removed. The neutral red quickly diffused through the vitelline membrane and coloured the embryo. This procedure was followed with 9 embryos, eggs then sealed and reincubated. Embryos were examined daily or every second day for eight days.

Using one or two drops of the 0.1% neutral red solution or using the neutral red-dyed agar, a single somite was removed from each of 44 chick embryos between stages 9 and 14. Additionally, a number of somite transplants were performed (15 "quail-to-chick" and 6 "chick-to-chick") using the neutral red marker system. Again these embryos were examined daily or every second day to eight days post-intervention.

Several control experiments were performed using black drawing ink (Pelikan, Gunther Wagner, or Speedball, Hunt) diluted 1:1 with Pannett and Compton's saline (with antibiotics) to visualize the embryo. In 57 chick embryos, windows were made, ink injected subendodermally, and the embryos reincubated. Twenty-eight single somite removals from chick embryos were performed again using the injected ink to see the outline of the embryonic structures. Each of these embryos was examined daily or every second day to eight days.

APPENDIX B

TISSUE PROCESSING FOR PARAFFIN EMBEDDING

The following protocol was used for tissue processing for histology:

1. 70% ethanol
2. 70% ethanol
3. 85% ethanol
4. 85% ethanol
5. 95% ethanol
6. 95% ethanol
6. Absolute ethanol
7. Absolute ethanol
8. Clearing agent*
9. Clearing agent*
10. Paraffin**
11. Paraffin**

* - Hemo-De (Fisher)

** - Tissue Prep Embedding Medium (Fisher)

Time for each step was between 15 minutes and 1 hour, depending upon the size of the tissue.

APPENDIX C

STAINING METHOD FOR THE FEULGEN REACTION

Procedure for DNA staining by the Feulgen method was as follows:

1. Xylene (three to four minutes)
2. Xylene (three to four minutes)
3. Absolute ethanol (three to four minutes)
4. Absolute ethanol (three to four minutes)
5. 95% ethanol (three to four minutes)
6. 85% ethanol (three to four minutes)
7. 70% ethanol (three to four minutes)
8. Distilled water (three minutes)
9. 1% Iodine in distilled water (three minutes)
10. Rinse in running tap water (three minutes)
11. 1% Sodium thiosulphate (three minutes)
12. Rinse in running tap water (three minutes)
13. Distilled water (three minutes)
14. 1N HCl (room temperature) (one minute)
15. 1N HCl (60°C) (five minutes)
16. Distilled water (three minutes)
17. Schiff reagent (Fisher) (45 minutes to one hour)
18. Potassium metabisulphite rinses (5ml of potassium metabisulphite and 5ml N-HCl in 90ml distilled water) (2 minutes each, 3 changes)
19. Dehydrate through to Xylene (two changes of each of 95% ethanol, absolute ethanol, and xylene).

APPENDIX D

TISSUE PROCESSING FOR SCANNING ELECTRON MICROSCOPY

The following regimen was used in preparation of the embryos for scanning electron microscopy:

1. Fix in 2.5% glutaraldehyde in 0.1M sodium phosphate buffer pH 7.4 at room temperature (approximately 12 to 18 hr)
2. 2 rinses in 0.1M sodium phosphate buffer (1 hr each)
3. Post-fix in 1% osmium tetroxide in 0.1M sodium phosphate buffer (1 hr)
4. 2 rinses in 0.1M sodium phosphate buffer (1 hr each)
5. Dehydrate through graded series of acetones (30 min in each of 25%, 60%, 85%, 95%, and 2 changes of 100% acetone)
6. Critical point drying from liquid CO₂ in a critical point dryer
7. Removal of the dried embryos from the filter paper rings and mounting of same with double-sided cellophane tape to SEM stubs
8. Sputter coating of the embryos with gold.

APPENDIX E

COMPUTER THREE-DIMENSIONAL RECONSTRUCTION TECHNIQUES

Using an IBM-3D program on an IBM PC with digitizing pad, the vertebral elements were traced with the stylus from the micrographs for every fifth section of the quail cell-containing vertebrae for embryo number 75-3. The quail cell regions were entered separately and stored in another file. The individual drawings were then compiled into a single drawing to represent the entire vertebra. The available software, however, did not permit hidden lines and the resulting graphics display was too extensively overdrawn to understand. Samples of these attempted reconstructions have been presented in Figure 44.

Figure 44. Computer three-dimensional reconstructions for C6 of embryo 75/3

